Prolongation of delayed antiarrhythmic protection by repeated cardiac pacing: Role of nitric oxide

PhD Thesis

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Summary

Right ventricular pacing induces both early and delayed protection against ventricular arrhythmias resulting from a prolonged coronary artery occlusion in dogs. The protection, however, is pronounced but transient. The early phase of protection disappears within 1-2 hours but returns 24 h later. This delayed antiarrhythmic protection starts to fade again 48 h after the pacing stimulus. The main purpose of the experiments discussed in this thesis was to examine whether this delayed phase of the protection can be prolonged by repeating the pacing stimulus at the time when the protection has waned (ie. 48 h after one period of pacing). Furthermore, we aimed to examine whether this protective effect of right ventricular pacing was mediated by nitric oxide and whether this involved induction of nitric oxide synthase.

Dogs were paced from the right ventricle four times for 5 min at a rate of 220 beats min^{-1} by means of a bipolar pacing electrode. The pacing stimulus was repeated 48 h later. At various times, 48, 72 and 96 h after the second pacing stimulus the dogs were reanaesthetised with chloralose and urethane, thoracotomised and subjected to 25 min occlusion of the left anterior descending coronary artery (LAD). Sham operated dogs served as controls.

To determine whether nitric oxide plays a role in the delayed protection against arrhythmias aminoguanidine (AG) and S-(2-aminoethyl)-methyl-isotiourea (AEST), selective inhibitors of the inducible nitric oxide synthase (iNOS), were administered in dogs subjected to one or two periods of cardiac pacing. We have also examined the time course of activation of nuclear factor κ-B (NFκB), a transcription factor involved in the expression of iNOS, in dogs subjected to right ventricular pacing (4x5 min) and in rabbits subjected to ischaemic preconditioning (4x5 min) by brief coronary artery occlusions.

Repeated right ventricular pacing prolonged the protection against ventricular arrhythmias which occurred during a 25 min occlusion of the LAD. Thus, 48 and 72 h after repeated cardiac pacing the number of ventricular premature beats and episodes of ventricular tachycardia were markedly suppressed, no dog fibrillated during the occlusion, and 50% and 73% of these dogs survived the reperfusion, respectively. When the time interval between the second pacing stimulus and the occlusion is extended to 96h the protection has already faded.

Administration of aminoguanidine either prior to cardiac pacing or just prior to coronary artery occlusion markedly attenuated the protection against ventricular arrhythmias 24h later. Similarly, in those repeatedly paced dogs which were treated with AEST before coronary artery occlusion the protection was abolished 72h after repeated pacing.

We have shown that NFκB is activated either by right ventricular pacing in dogs or by ischaemic preconditioning in rabbits. The time course of the activation of NFκB differs in these two animal models. In dogs right ventricular pacing resulted in activation of NFκB 1 h after pacing, but in rabbits ischaemic preconditioning activated the transcription factor 10 min after the last preconditioning occlusion.

We can conclude that repeating the pacing stimulus at a time when the protection has already faded prolongs the protection against ischaemia-reperfusion induced ventricular arrhythmias for 72 hours but it has waned 96 h later. Nitric oxide may play a role in the delayed antiarrhythmic protection since administration of aminoguanidine or AEST, selective inhibitors of the inducible nitric oxide synthase, profoundly attenuates the delayed phase of the protection induced either by single or repeated pacing stimulus.

List of contents

List of publications

Full papers

I. Kis, A., Végh, Á, Papp, J. Gy., Parratt, J.R. Repeated cardiac pacing extends the time during which canine hearts are protected against ischaemia-induced arrhythmias : role of nitric oxide. *J. Mol. Cell. Cardiol.* **31**: 1129-1141, 1999. Impact factor: 3.255

II. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Pacing-induced delayed antiarrhythmic protection is attenuated by aminoguanidine in dogs. *Br. J. Pharmacol.* **127**: 1545-1550, 1999. Impact factor: 3.619

III. Kis, A., Végh, Á., Papp, J. Gy., Parratt, J.R. Simultaneous blockade of the cyclooxygenase and L-arginine-nitric oxide pathways prevents the antiarrhythmic effects of classical preconditioning. *Exp. Clin. Cardiol.* **2**: 112-118, 1997. Impact factor: -

Book chapter

IV. Végh, Á., Kis, A., Papp, J. Gy., Parratt, J.R. Early and delayed protection against ventricular arrhythmias induced by preconditioning. In: *Ischaemic Heart.* Eds.: S. Mochizuki and M. Nagano, Kluwer Academic Publisher, 279-303, 1997.

Abstracts

V. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Dual blockade of the cyclo-oxygenase and L-arginine-nitric oxide pathways prevents the antiarrhythmic effect of preconditioning. *J. Mol. Cell. Cardiol.*, **27**: A159, 1995.

VI. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Antiarrhthmic effects of preconditioning are prevented by a dual blockade of cyclooxygenase and L-arginine-nitric oxide pathways. *Cardiol. Hung.* Suppl. **1** , 40, 1995.

VII. Kis, A., Kaszala, K., Végh, Á., Papp, J.Gy., Parratt, J.R. Repeated pacing widens the time window of delayed protection against ventricular arrhythmias in dogs. *J. Mol. Cell. Cardiol.*, **28**: A59, 1996.

VIII. Kis, A., Kaszala, K., Végh, Á., Papp, J.Gy., Parratt, J.R. Prolongation of the Second Window of Protection by repeated cardiac pacing in dogs. *Cardiol. Hung.* Suppl. **1**, P59, 1996.

IX. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Repeated pacing markedly prolongs the delayed antiarrhythmic protection in anaesthetised dogs. *J. Mol. Cell. Cardiol.*, **29**: ASa62, 1997.

X. Kis, A., Végh, Á, Papp, J.Gy., Parratt, J.R. Repeated pacing widens the time window of delayed antiarrhythmic protection in canine. *J. für Kardiol.* 1997/2.

XI. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Pacing-induced antiarrhythmic protection is attenuated by aminoguanidine in dogs. *J. Mol. Cell. Cardiol.* **30**: A280, 1998.

XII. Kis, A., Végh, Á, Papp, J.Gy., Parratt, J.R. Role of nitric oxide in the delayed antiarrhythmic protection induced by rapid cardiac pacing in anaesthetised dogs. *Cardiol. Hung.* Suppl. **98/1**. 1998.

XIII. Végh, Á., Kis, A., Papp, J.Gy., Parratt, J.R. Repeated cardiac pacing prolongs delayed protection against ventricular arrhythmias in canine. *J. Am. Coll. Cardiol.* **31** (Suppl. C): A1726, 1998.

XIV. Kis, A., Végh, Á, Papp, J.Gy., Parratt, J.R. Repeated cardiac pacing prolongs protection against ischaemia-induced ventricular arrhythmias in anaesthetised dogs. *J. Physiol.* **506**: 56P, 1998.

XV. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Delayed antiarrhythmic protection induced by repeated pacing is abolished by S-(2-aminoethyl)-methyl-isothiourea, a selective inhibitor of iNOS in dogs. *J. Mol. Cell. Cardiol.* **31**: A63, 1999.

XVI. Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. Delayed cardioprotection induced by rapid cardiac pacing is attenuated by aminoguanidine. *Fund. Clin. Pharmacol.* **13**(Suppl. 1): PT125, 1999.

1. Introduction

Ischaemic heart disease, myocardial infarction and sudden cardiac death are among the main causes of the high mortality rate in developed countries. Certainly, genetic factors contribute to the development of these but the risk is increased by other factors, for example smoking, cholesterol rich diet and a sedentary lifestyle. Once disease has developed the only possibility is treatment for the rest of life. At present we are not aware of a drug which completely obviates this condition.

Myocardial ischaemia is a pathologic state of the myocardium resulting from an insufficient blood supply together with a concomitant reduction in oxygen supply. The consequences of myocardial ischaemia depend on the duration, as well as on the degree, of an ischaemic insult (eg. the coronary artery is occluded totally or partially). The inadequate blood (and oxygen) supply to the myocardium results in several metabolic changes, ie. a change from aerobic glycolysis to anaerobic glycolysis leading to an accumulation of lactate, a decrease in pH, an increase in inorganic phosphates and depletion of ATP. These changes influence the function of the contractile elements, and of several proteins as well as ion channels in the membranes (Na^+/K^+ ATPase, Na^+/H^+ exchanger, K^+_{ATP} channel) resulting in complete disturbance of the homeostasis of the cell. When the occlusion of the coronary artery is prolonged (30-40 min), despite restoration of the blood flow, these alterations become irreversible which ultimately lead to myocardial cell death. If the duration of myocardial ischaemia is shorter (ie. 5-15 min) the risk of the generation of fatal ventricular arrhythmias and development of necrosis is reduced although the contractile function of the heart may remain suppressed for a longer period (even days) after complete restoration of myocardial blood flow.

1.1. Discovery and characteristics of ischaemic preconditioning

Despite the high risk for the development of ischaemic heart disease and its often fatal consequences, intrinsic protective pathways exist to counteract them. It was more than a decade ago when Murry and his colleagues reported that in anaesthetised dogs sublethal brief periods of ischaemia, which did not in themselves lead to irreversible injury, paradoxically protected the heart from a subsequent more prolonged ischemic insult. They termed the phenomenon "ischaemic preconditioning" (1). They started from the hypothesis that if the

coronary artery occlusion was repeated within a short time (4x5 min with 5 min reperfusion intervals in between) this would lead to a cumulative depletion of the ATP and high energy stores, enhance the development of myocardial necrosis. Surprisingly, they found preservation of ATP and a delay in cell death during the prolonged (40 min) occlusion of the same coronary artery. Thus, myocardial infarct size expressed as the percentage of the area at risk was reduced from 30% in controls subjected only to a 40 min occlusion to 7% in preconditioned dogs. This marked reduction in infarct size was independent from changes in transmural collateral flow.

Ischaemic preconditioning protects the heart against other severe consequences of myocardial ischaemia such as contractile dysfunction or life-threatening ventricular arrhythmias. For example, there is a great deal of evidence that preconditioning enhances the recovery of contractile function following reperfusion of the ischaemic myocardium (2). There is even earlier evidence that short periods of ischaemia protect against ventricular arrhythmias resulting from coronary artery occlusion. In 1977, Gülker and colleagues showed that in anaesthetised dogs the reduction of the ventricular fibrillation threshold was markedly attenuated following repeated coronary artery occlusions (3). In 1983, Barber showed that a single 5 min LAD occlusion significantly decreased the number of ventricular ectopic beats resulting from a second period of occlusion of the same duration if the reperfusion period between the occlusions was 3 min (4). And then in 1987, Shiki and Hearse demonstrated in anaesthetised rats that similar short periods (5 min) of coronary artery occlusion reduced the incidences of reperfusion induced ventricular tachycardia and fibrillation; ischaemia induced arrhythmias were not examined (5). Later, in 1990 Végh and colleagues reported, in anaesthetised dogs, that the severity of ventricular arrhythmias occurring during a 25 min prolonged ischaemia and subsequent reperfusion was significantly reduced if the dogs had been subjected to ischaemic preconditioning by two 5 min occlusions, 20 min previously (6)..

Several studies have revealed that ischaemic preconditioning of the heart is a general phenomenon since tolerance against the severe consequences of myocardial ischaemia can be induced in all species examined so far; ie. dogs (1, 6, 7), rats (7-10), pigs (11, 12), rabbits (13-15), and humans (16, 17). Of great importance is the finding that in patients repeated balloon inflations during percutaneous transluminal coronary angioplasty (PTCA) results in similar protective effects as ischaemic preconditioning. Thus, the pain, lactate production and ST-

segment changes were markedly less during the second balloon inflation compared to the first indicating that the first ischaemic episode rapidly induced protection, ie. by the time of the second ischaemic episode (17).

There are other sublethal (ischaemic and non-ischaemic) stress stimuli which may lead to protection of the heart. These include right ventricular pacing (18, 19), myocardial stretch (20), cyclic flow variations (21), hypoxia (22, 23), heat stress (24) and exercise (25, 26).

1.2. Time dependent characteristic of ischaemic preconditioning

The cardioprotection associated with preconditioning is extremely powerful but unfortunately transient. The degree of the protection depends on the number and duration of occlusion periods (5, 27, 28) and on the time interval elapsing between the preconditioning stimulus and the prolonged ischaemic insult (7, 28). If the time interval between the preconditioning stimulus and the prolonged coronary artery occlusion is extended to 30 min or 1 h the protection, for example against ischaemic damage or ventricular arrhythmias, is largely attenuated or abolished (7, 29-33). For example, rapid cardiac pacing (4x5 min) in dogs induces marked antiarrhythmic protection when myocardial ischaemia is initiated 5 min after pacing but the protection is not apparent if the time interval between the pacing stimulus and the occlusion is extended to 15 min, 1 h or 6 h (30). In pigs, rapid ventricular pacing (30 min) induces protection against ischaemic damage if the prolonged ischaemia is initiated immediately but it is slightly reduced 15 min later (34). Similarly, in conscious rabbits cardiac pacing results in protection against consequences of a subsequent ischaemic episode, as shown by reductions in ST-segment elevation and in the increase in left ventricular end-diastolic pressure (35). The reduction in the severity of ischaemia is completely lost if the time interval between the preconditioning stimulus and the subsequent ischaemia is extended to 30 min (35).

Interestingly, in anaesthetised rabbits, the commencement of a second 5 min occlusion at the time when the protection resulting from a previous preconditioning stimulus is lost can restore the protection against infarction (36). In contrast, repeating the preconditioning stimulus in pigs 1 h later fails to renew the protection against ischaemic damage (29). It is possible to enhance this fading protection pharmacologically. In rabbits the cardioprotection induced by short coronary artery occlusions waned after 2 h but could be enhanced at this time by the administration of acadesine, a drug which is believed to act by increasing the level of adenosine in the myocardium (32, 33).

The cardioprotection which disappears within 1-2 hours, and which is termed now as "classical preconditioning", returns 24 h after the preconditioning stimulus. This delayed phase of protection was first reported by Yamashita and colleagues in 1992 (37) and later by Kuzuya and colleagues in 1993 (38). In their studies dogs were subjected to ischaemic preconditioning and the extent of the infarction was examined immediately and 3 h, 12 h and 24 h later. They found that there was a significant reduction in infarct size when the sustained occlusion followed the preconditioning stimulus either immediately or 24 h later but not at intermediate times (3 h and 6 h). In the same year Marber and colleagues reported that the extension of myocardial necrosis was significantly smaller in rabbits subjected to ischaemic preconditioning or heat stress 24 h previously (39). Similarly, in anaesthetised dogs the severity of ventricular arrhythmias during a 25 min coronary artery occlusion and following reperfusion was significantly reduced if the dogs had been subjected to right ventricular pacing 20-24 h previously (30, 40, 41). In conscious rabbits right ventricular pacing (ten times for 5 minutes) induced protection against consequences of a subsequent test (pacing-induced) ischaemia which was present 24 and 48 h after pacing but not 72 h later (35). Delayed protection induced by short coronary artery occlusions against ischaemic damage in anaesthetised rabbits was more prolonged; it lasted for 72 hours (42-44). Similarly, preconditioning in conscious pigs decreased the degree of contractile dysfunction (myocardial stunning) 24 h later; this protection also lasted for 72 h (45, 46). In dogs the pronounced protection against ischaemia and reperfusion induced ventricular arrhythmias faded by 48 h and was lost 72 h after the pacing stimulus (30).

1.3. Possible mechanisms involved in the cardioprotective effects of ischaemic preconditioning

Since the protection of the heart to a sustained ischaemic insult afforded by various sublethal preconditioning stimuli is so powerful it would be important to explore the underlying mechanisms in the hope of developing new therapeutic interventions. Although several hypotheses have attempted to explain both the early and the delayed cardioprotective effects of preconditioning the precise mechanisms of the protection are still unclear. There is a strong evidence that the release of endogenous myocardial protective substances such as adenosine, bradykinin, prostacyclin and nitric oxide deriving from the vascular endothelium and also from cardiac myocytes are involved both in the early and the delayed phase of cardioprotection (47).

One of the endogenous substances which is released abundantly from the ischaemic myocardium is adenosine. Several studies have demonstrated the cardioprotective effects of adenosine in the ischaemic heart. Thus, adenosine can suppress arrhythmias (48, 49), limit infarct size (14) and decrease contractile dysfunction (50). Adenosine most probably plays a role as a trigger of the protection against ischaemic damage via activation of A_1 receptors (14, 51). The evidence for the involvement of A_1 receptors in this form of cardioprotection comes from studies which demonstrated that the infarct size resulted from prolonged ischaemia was markedly reduced by prior administration of an A_1 selective agonists such as R-phenylisopropyl adenosine (R-PIA) or 2-chloro-N6-cyclopenthyl-adenosine (CCPA) (52). Adenosine infusion in blood-perfused rabbit hearts mimicked the effect of ischaemic preconditioning against ischaemic damage; the limitation of infarct size was similar to that found after ischaemic preconditioning (53). However, the "in vivo" experiments were not able to prove that adenosine plays a trigger role in the infarct size limiting effect of preconditioning (54).

The other endogenous protective substance which might be involved in the cardioprotective effects of ischaemic preconditioning is bradykinin. It is well established that bradykinin is generated and released in the very early stage of ischaemia by activation of an acid optimum kininogenase enzyme (55). Furthermore, bradykinin infused into a small branch of the LAD markedly reduced the ischaemia-induced ventricular arrhythmias in anaesthetised dogs (56). There is some evidence that bradykinin is involved in the protective effect of ischaemic preconditioning since icatibant (HOE140), a selective antagonist of bradykinin at B_2 receptors, abolished the antiarrhythmic effect of preconditioning induced either by short coronary artery occlusions or cardiac pacing in anaesthetised dogs (57, 58). Bradykinin proved to be also involved in the infarct size limiting effect of ischaemic preconditioning and this effect could be reversed by icatibant (59, 60).

There is some evidence that bradykinin stimulates the generation of nitric oxide and prostacyclin (61, 62). The antiarrhythmic effect of bradykinin was largely attenuated by inhibiting the generation of nitric oxide with L-nitroarginine methyl esther (L-NAME) which indicates a bradykinin-induced nitric-oxide mediated mechanism in the antiarrhythmic effect of preconditioning (63). Furthermore, inhibition of the formation of nitric oxide with L-NAME (64) and cGMP with methylene blue (65) markedly attenuates the antiarrhythmic protection induced by ischaemic preconditioning. According to these findings a hypothesis for the antiarrhythmic effect of ischaemic preconditioning is that bradykinin triggers the release of

nitric oxide from the vascular endothelium which diffuses into the cardiac myocytes and increases the level of cGMP level by stimulating the soluble guanylate cyclase enzyme.

1.4. Aims of the studies

1. The main objective of the studies presented in this thesis was to determine whether the marked delayed antiarrhythmic protection could be prolonged by repeating the pacing stimulus at the time when the protection from the previous preconditioning stimulus had faded.

2. A second series of experiments aimed to determine whether the mechanism of this marked early and delayed antiarrhythmic protection involves nitric oxide and prostacyclin. For this purpose experiments were designed to investigate:

a, the involvement of nitric oxide and prostacyclin in classical preconditioning by simultaneous blockade of the L-Arg/NO and cyclooxygenase pathways,

b, the involvement of nitric oxide in the delayed antiarrhythmic protection induced by „single" or repeated periods of cardiac pacing by administration of selective inhibitors of the inducible nitric oxide synthase,

c, the time course of the activation of nuclear factor κ-B (NFκB), a transcription factor which is involved in the expression of iNOS, both in dogs subjected to right ventricular pacing and in rabbits subjected to ischaemic preconditioning by coronary artery occlusions.

2. Materials and methods

2.1. Animals

Experiments were carried out in adult, mongrel dogs of both sexes with a body weight in excess of 17 kg. The dogs were allowed to access to food and water *ad libitum* until starting the experiments. All animals received humane treatment according to the *Guide for the care and use of laboratory animals* published by National Institute of Health of the USA (1985) and by a local university policy.

2.2. Surgical preparations

Dogs were anaesthetised with a mixture of chloralose and urethane (60 mg kg^{-1} and 200 mg kg^{-1}, respectively), ventilated with room air using a Ugo respirator (Hugo Sachs Elektronik,

Germany) at a sufficient rate and volume to maintain the arterial blood gases and pH within normal limits (pO_2: 80-105 Hgmm, pCO_2: 35-54 Hgmm, pH: 7,36-7,44). The body temperature was monitored from the oesophagus and maintained at 37±0.5 ^{o}C by using a heating pad. The animals were thoracotomised in the fifth intercostal space and the left anterior descending coronary artery (LAD) was prepared for occlusion proximal to the first main diagonal branch (Figure 1). Myocardial ischaemia was induced by a 25 minute occlusion of the LAD after which the occlusion was rapidly released. In some experiments drugs were infused intracoronarily into a small side branch of the LAD, proximal to the occlusion site (Figure 1). To measure the basic heamodynamic parameters polyvinyl catheters were introduced into the right femoral artery for monitoring the arterial blood pressure (systolic: SABP, diastolic: DABP), to the left ventricle via the left carotid artery to measure the left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures and the maximal positive and negative dP/dt, and into the right femoral vein for administration of anaesthetics and drugs. Haemodynamic parameters were measured by means of pressure transducers (P23XL, Hugo Sachs Elektronik, Germany). To evaluate the severity of myocardial ischaemia a composite electrode was sutured to the risk area of the left ventricle; this composite electrode also contained four unipolar electrodes (66, 67). By means of this electrode changes in epicardial ST segment elevation and the degree of inhomogeneity of electrical activation were recorded. The composite electrode gives a summarised recording of R waves from 30 epicardial measuring points. In the normal adequately perfused and oxygenated heart these sites in the left ventricular wall are activated at almost the same time giving rise to a large spike on the ECG. During ischaemia the adjacent fibres are not activated simultaneously and, due to the inhomogeneity of conduction, fractionation and widening of the R wave appears. The inhomogeneity of electrical activation is expressed as the greatest delay (ms) in the activation within the ischaemic area under the composite electrode. All of these parameters, together with the limb lead electrocardiogram, were recorded on a Graphtec Thermal Array Recorder (Hugo Sachs, Germany).

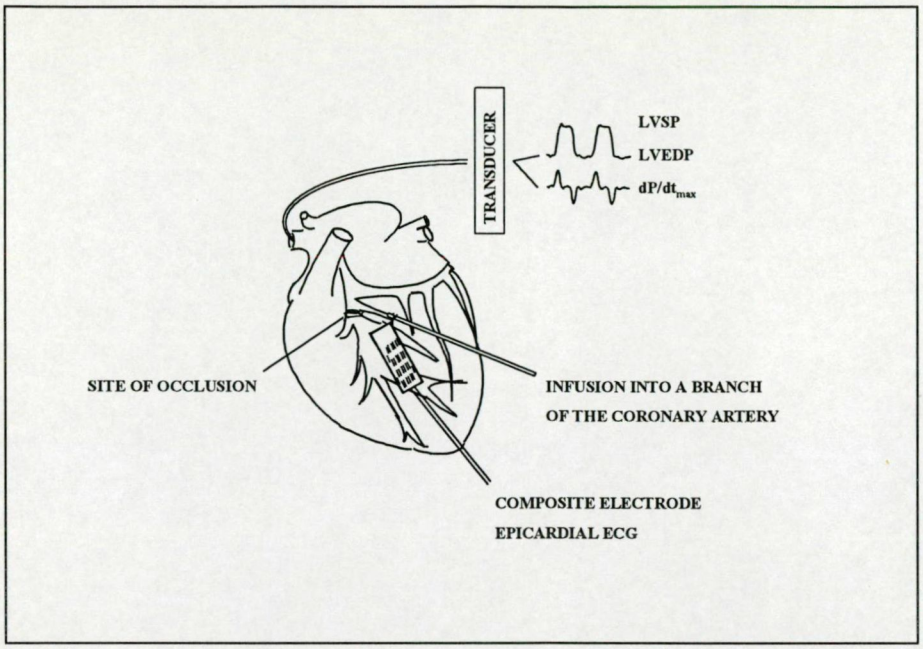

Figure 1. The experimental model in anaesthetised dogs for the measurement of haemodynamic parameters, changes in epicardial ST-segment elevation and degree of inhomogeneity of electrical activation and for the assessment of ventricular arrhythmias.

2.3. Evaluation of ventricular arrhythmias

Ventricular arrhythmias were evaluated by a method largely based on the principles laid down by the Lambeth' Convention (68). Thus, the number of ventricular premature beats (VPBs) was determined without distinction between couplets and salvos which were assessed as single ventricular ectopic beats. The number of episodes of ventricular tachycardia (VT) in each dog, the incidences of ventricular tachycardia and ventricular fibrillation (VF) were also determined both during the occlusion period and following the initiation of reperfusion. Those dogs which fibrillated during occlusion or reperfusion were not defibrillated. Survival indicates those dogs which were in sinus rhythm without any ischaemic alterations on the epicardial or limb lead ECGs for at least 10 minutes after reperfusion.

2.4. Determination of the area at risk

At the end of the experiments the heart was excised and the LAD suture was again tightened. Methylene blue dye was infused into the occluded coronary artery distal to the site of occlusion. The risk (dyed) area was expressed as the percentage of the mass of the left ventricular wall together with the septum.

2.5. Experimental protocols

2.5.1. Protocol to examine the role of nitric oxide and prostacyclin in classical preconditioning

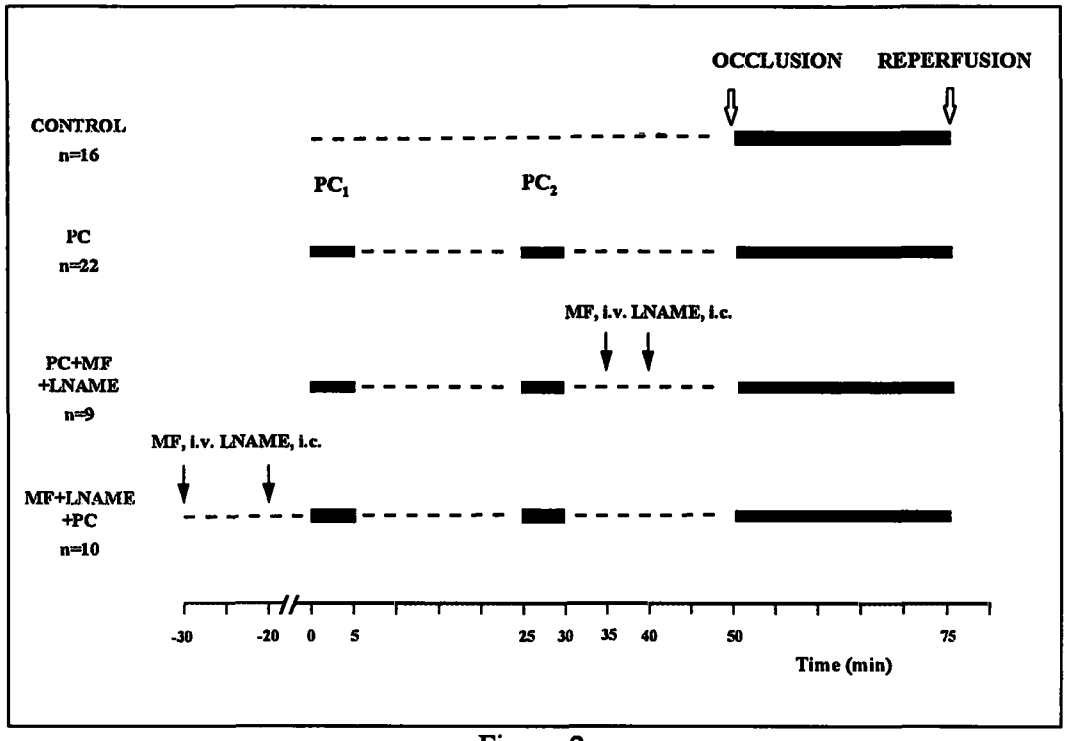

Figure 2.

Dogs were preconditioned by two 5 min occlusion of the LAD with 20 min reperfusion period in between (PC, n=22). Myocardial ischaemia was induced by a 25 min occlusion of the same artery 20 min after the second preconditioning occlusion. Control dogs were subjected only to a 25 min coronary artery occlusion followed by reperfusion (n=16). In one group of preconditioned dogs sodium meclofenamate (MF, a cyclooxygenase (COX) inhibitor) was administered intravenously in a dose of 2 mg kg^{-1} followed by infusion of L-nitroarginine methyl esther (L-NAME, a nitric oxide synthase (NOS) inhibitor) intracoronarily in a dose of 5 mg kg^{-1} 30 min and 20 min before preconditioning, respectively (n=9). In one group of preconditioned dogs meclofenamate and L-NAME were administered 15 and 10 min before the prolonged ischaemic insult, respectively (n=9).

2.5.2. Protocol for the determination of the role of NO in delayed protection induced by a single cardiac pacing

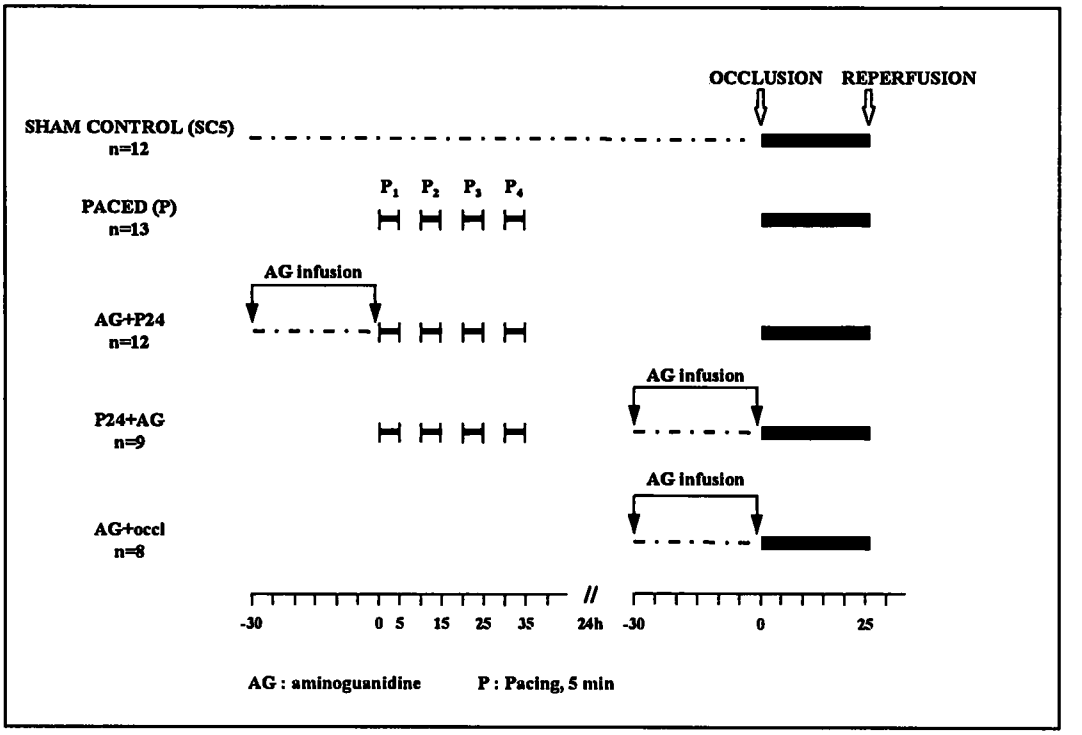

Figure 3.

For the commencement of cardiac pacing a bipolar pacing electrode (Cordat F4) was introduced under light pentobarbitone anaesthesia into the right ventricle via the right jugular vein. The correct position of the electrode was confirmed by recording the endocardial electrocardiogram from the pacing electrode and monitored continuously during the procedure. Dogs were paced four times for 5 minutes at a rate of 220 beats min^{-1}. Changes in mean arterial blood pressure (MABP) were registered by means of a catheter introduced into the left carotid artery. During pacing ST-segment changes on the endocardial ECG and limb lead ECG were also recorded. 24 h later, these preconditioned dogs (P, n=13) were re-anaesthetised with a mixture of chloralose and urethane, thoracothomised and the LAD was occluded for 25 minutes followed by rapid reperfusion. In some paced dogs aminoguanidine (AG) was administered intravenously in a dose of 50 mg kg^{-1} either 30 minutes prior to pacing (AG+P24, n=12), or 30 minutes prior to the prolonged occlusion of the LAD but 24 h after right ventricular pacing (P24+AG, n=9). The sham-operated dogs served as controls. In these 12 dogs (SC5) the bipolar pacing electrode and the polyvinyl catheter were introduced into the right ventricle and the left carotid artery but these dogs were not paced. 24 hours later these dogs were re-anaesthetised and subjected to myocardial ischaemia followed by reperfusion. Another 8 dogs which were not paced but given aminoguanidine 30 minutes before coronary

artery occlusion served as the aminoguanidine control group to determine whether AG itself modified the severity of ventricular arrhythmias.

2.5.3. *Protocol for evaluation of the prolongation of delayed antiarrhythmic protection induced by repeated cardiac pacing; the role of NO*

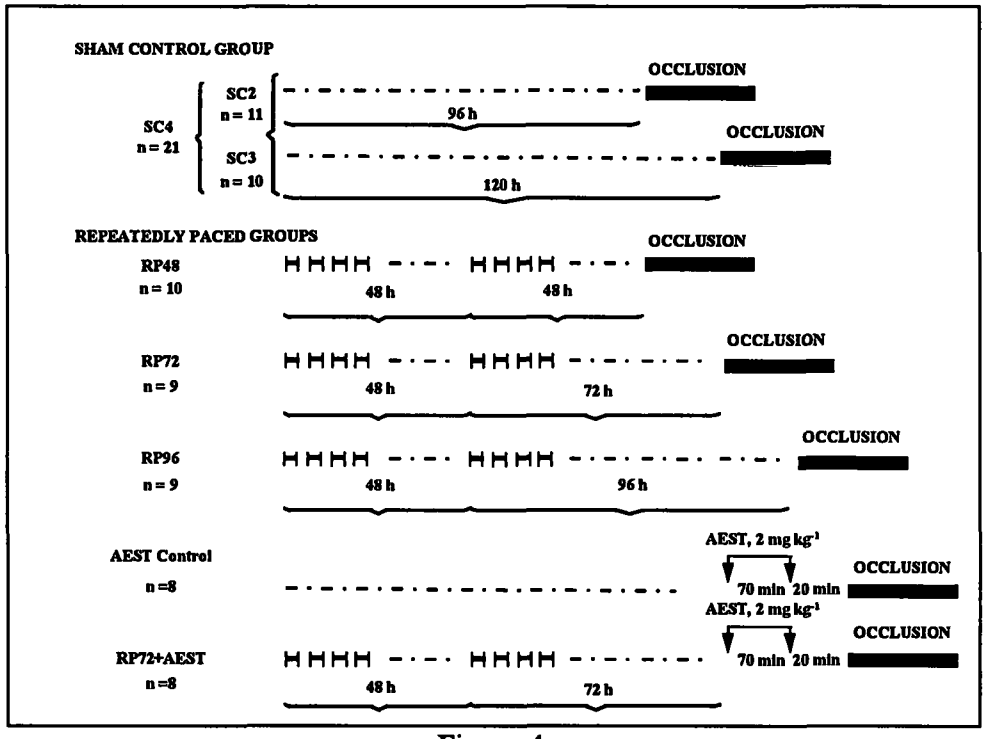

Figure 4.

This study was designed to examine whether the delayed antiarrhythmic protection resulting from right ventricular pacing could be prolonged by repeating the pacing stimulus at a time when the protection was no longer apparent, ie. 48h after one period of cardiac pacing (30). In these experiments dogs underwent sterile surgical intervention under light pentobarbitone anaesthesia. Then a bipolar pacing electrode was introduced into the right ventricle and a polyvinyl catheter was led into the left carotid artery to measure changes in mean arterial blood pressure during pacing. The animals were paced four times for 5 min at a rate of 220 beats min^{-1} and left to recover from anaesthesia for 48 h. After recovery the dogs were repaced in the same manner as described above and left to recover for various time intervals. 48, 72 or 96 h after the second pacing procedure dogs were re-anaesthetised with a mixture of chloralose and urethane, thoracotomised and the LAD was prepared for occlusion. Myocardial ischaemia was induced by a 25 min occlusion of the coronary artery followed by reperfusion. Under pentobarbitone anaesthesia sham-operated control dogs were subjected to the same surgical interventions but the dogs were not paced, although they were re-anaesthetised with

pentobarbitone 48h later. After 48h or 72h recovery they were subjected to coronary artery occlusion for 25 min.

In the second part of the study, in repeatedly paced dogs S-(aminoethyl)-methyl isothiourea (AEST, 2 mg kg^{-1}) was given starting the infusion 90 min before occlusion of the LAD. To determine the effect of AEST on ventricular arrhythmias 8 sham-operated dogs, without repeated pacing, were given AEST 90 min before coronary artery occlusion.

2.6. Electromobility shift assay for determinaion of the activation of nuclear factor κ-B

Experiments were designed to determine whether nuclear factor κ-B (NFκB), the transcription factor involved in the expression of inducible nitric oxide synthase, was activated after preconditioning induced by right ventricular pacing. These studies were performed at the Department of Pharmacology and Pharmacotherapy of the Albert Szent-Györgyi Medical University, in Szeged and at the Hatter Institute and Centre for Cardiology of University College London, in London. Dogs were paced four times for 5 min through the right ventricle by means of a pacing electrode. At various times, 15 min, 1h and 24h later, the hearts were excised and samples from the left ventricle were obtained, frozen in liquid nitrogen and stored at -80°C until used. In another series of the experiments male rabbits were used with a body weight of 2.7 kg. These were preconditioned by four periods of 5 min occlusion of the LAD separated by 10 min reperfusion periods. 10 min, 1h, 3h after last reperfusion the hearts of these animals were excised and samples from the risk area of the left ventricle were frozen in liquid nitrogen and stored at -80°C until used. Nuclear proteins were purified by the method of Schreiber with some modifications (69). Briefly, 0.1-0.15 g of heart tissue were homogenised and centrifuged in 1 ml ice-cold PBS solution. The supernatant was discarded and the pellet was suspended in 400 µl ice-cold buffer A (10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethansulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pH=7.9) and incubated for 15 min on ice. Nonidet P40 was added to the homogenate, vortexed for 45 s, incubated for 5 min and centrifuged at 14000 rpm for 1 min at 4 °C. The supernatant with the cytosolic protein fraction was stored at -80°C and the pellet of nuclei was resuspended in 400 µl ice-cold buffer C (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH=7.9) and rocked on a shaking platform for 30 min on ice. The samples were then centrifuged for 15 min at 14.000 rpm at 4 °C. The supernatant was collected as the nuclear protein fraction and stored at -80 °C.

Activation of NFκB was examined by the ElectroMobility Shift Assay (EMSA). Nuclear proteins (20 μg) were combined with poly dIdC (0.2 μg μl^{-1}) to reduce the non-specific DNA binding, binding buffer (4% glycerol, 1 mM EDTA, 10 mM Tris pH=7.5, 0.1 M NaCl, 10 μg BSA, 5 mM DTT, 1 mM $MgCl_2$) and labelled oligonucleotide (5' AGT TGA GGG GAC TTT CCC ACG C 3') in a total volume of 15 μl, incubated for 30 min at room temperature and resolved on a 5% non-denaturing polyacrilamide gel. This gel was pre-run for 3 h at room temperature in 0.25X tris(hydroxymethyl)aminoethane/ boric acid/ EDTA buffer (TBE) at 125V. After running for 3h the gels were dried at 80 ^{o}C without vacuum for an hour and exposed to Kodak film at -70 ^{o}C. Oligonucleotide was 5' end-labelled as dsDNA with [γ-^{32}P]ATP using T4 polynucleotide kinase. In competition assay unlabelled cold probe was also added in excess to the binding reaction and it was incubated for 10 min before adding labelled probe to the reaction.

2.7. Statistical analysis

The data are expressed as mean ± s.e.m. and differences between means were compared by analysis of variance (ANOVA) or by Student's t test. A one-way ANOVA was used to compare differences in haemodynamic parameters. Mann-Whitney rank sum test was used for analysis of VPBs and episodes of VT, and Fisher exact test was used for comparison of the incidences of VT, VF and survival. Differences between groups was considered significant when $P < 0.05$.

2.8. Chemicals

Nembutal was purchased from Phylaxia-Sanofi, α-Chloralose, Uretane, Sodium Meclofenamate, L-Nitro Arginine Methyl Esther, Aminoguanidine Hemisulphate, Methylene blue dye, EDTA, EGTA, DTT, PMSF from Sigma, S-(aminoethyl)-Methyl Isothiourea a kind gift from Csaba Szabó, NFκB oligonucleotide from Santa Cruz, T4 polynucleotide kinase from Promega, [γ-^{32}P]ATP, aprotinin, leupeptin from ICN, HEPES, KCl, NaCl from BDH laboratory supplies.

3. Results

3.1. Prolongation of delayed antiarrhythmic protection by repeating the pacing stimulus

3.1.1. Haemodynamic and electrocardiographic effects of repeated cardiac pacing

Right ventricular pacing increased heart rate (HR) from a mean of 169±4 beats min^{-1} to 220 beats min^{-1} and transiently decreased (approximately 35%) mean arterial blood pressure (MABP) immediately after initiation of the pacing stimulus (Table 1). When the pacing stimulus was repeated 48 hours later a significant decrease was found in baseline MABP and HR, but changes in these parameters were similar on day three (Table 1).

Table 1. Changes in heart rate and mean arterial blood pressure during pacing

	1. day			3. day		
	before pacing	after pacing	change	before pacing	after pacing	change
HR (beats min^{-1})	169±4	220±0	51±4 *	155±4 †	220±0	75±8 *
MABP (mmHg)	141±4	112±6	29±4 *	110±4 †	82±4	18±5 *

* $P < 0.05$ vs. baseline † $P < 0.05$ vs. 1. day baseline

Table 1. Haemodynamic parameters during pacing on day one and day three in repeatedly paced dogs. All individual data in the these groups were combined into one group because there was no difference between them. HR: heart rate (beats min^{-1}), MABP: mean arterial blood pressure (mmHg).

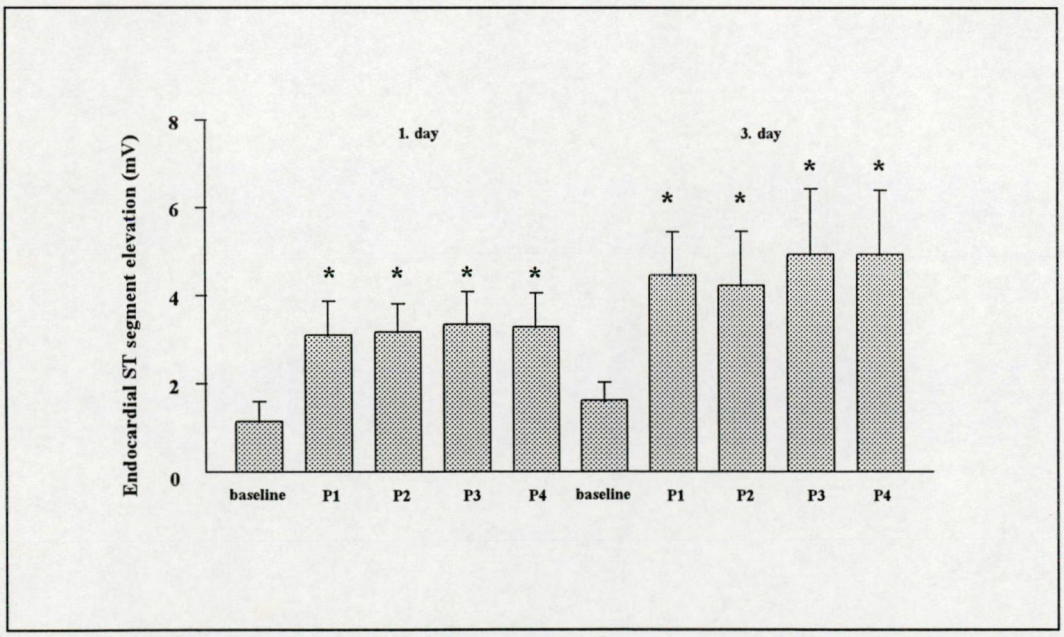

*Figure 5. Changes in endocardial ST segment elevation recorded from the right ventricle (mV) immediately after cessation of pacing in repeatedly paced groups on day 1 and day 3. * $P<0.05$ vs. baseline.*

Rapid cardiac pacing resulted in a transient, but significant, elevation in the endocardial ST-segment (a sign of ischaemia induced by pacing) from a mean baseline of 1.1±0.5 mV to 3.2±0.6 mV after cessation of the first pacing stimulus, to 3.3±0.8 mV after both second and third pacing stimuli, and to 3.3±0.5 mV after the fourth pacing stimulus. ST-segment elevation was slightly higher when the pacing stimuli were repeated 48 h later; it was increased from 1.6±0.4 mV to 4.5±1 mV, 4.2±1.2mV, 4.9±1.5mV, respectively after termination of the pacing stimuli.

All haemodynamic and electrocardiographic changes returned to the baseline values within 1-2 minutes after cessation of pacing.

3.1.2. Haemodynamic effects of coronary artery occlusion

Coronary artery occlusion resulted in slight or significant decreases in systolic, diastolic and mean arterial blood pressures, left ventricular systolic pressure and in positive and negative dP/dt and an increase in left ventricular end-diastolic pressure within a few minutes of the initiation of the coronary artery occlusion (Table 2). Changes in these haemodynamic parameters were similar in sham-operated dogs and in those dogs that had been paced repeatedly 48, 72 or 96 h previously.

Table 2 Haemodynamic parameters during LAD occlusion in chloralose and urethane anaesthetised dogs

	Sham Control		RP48		RP72		RP96	
	baseline	change	baseline	change	baseline	change	baseline	change
SABP (mmHg)	131±6	-10±3 *	139±5	0.5±2	149±9	-11±4 *	118±4	-7±4
DABP (mmHg)	89±4	-7±3 *	93±4	0.2±2	88±7	-5±3	76±5	-9±3 *
MABP (mmHg)	103±4	-8±3 *	109±3.5	0.3±2	109±7	-7±3	90±4	-8±2 *
LVSP (mmHg)	128±7	-11±4 *	136±6	1±2	131±11	-7±3 *	112±4	-5±4
LVEDP (mmH)g	9±2	-3.4±2	7±2	5±1.5 *	11±2	0.8±2	8±1	5±1 *
+dP±dt (mmHg s^{-1})	2237±214	-360±219	1998±163	-144±80	2936± 265	-355±304	2820±454	-563±348
-dP±dt (mmHg s^{-1})	3004±267	-326±107 *	2247±313	-142±98	3295±191	-533±216 *	2850±126	-343±121
HR (beats min^{-1})	148±5	5.4±1.5 *	164±9	4.2±1.4 *	146±10	0.6±1	149±5	0.6±1

Table 2. Changes in haemodynamic parameters during coronary artery occlusion in sham control and repeatedly paced dogs (RP48, RP72, RP96). SABP: systolic arterial blood pressure, DABP: diastolic arterial blood pressure, MABP: mean arterial blood pressure, LVSP: left ventricular systolic pressure, LVEDP: left ventricular end diastolic pressure, HR: heart rate, +dP/dt: positive left ventricular dP/dt, -dP/dt: negative left ventricular dP/dt. $P<0.05$ vs. baseline, data are expressed as mean±sem.

3.1.3. Ischaemia and reperfusion induced ventricular arrhythmias in sham control dogs and in dogs subjected to repeated pacing

Right ventricular pacing results in a marked protection against ischaemia and reperfusion-induced ventricular arrhythmias, when the LAD was occluded 24 h after the pacing stimulus (30, 40, 41). However, this protection has faded after 48 h or is lost after 72 h (Figure 6).

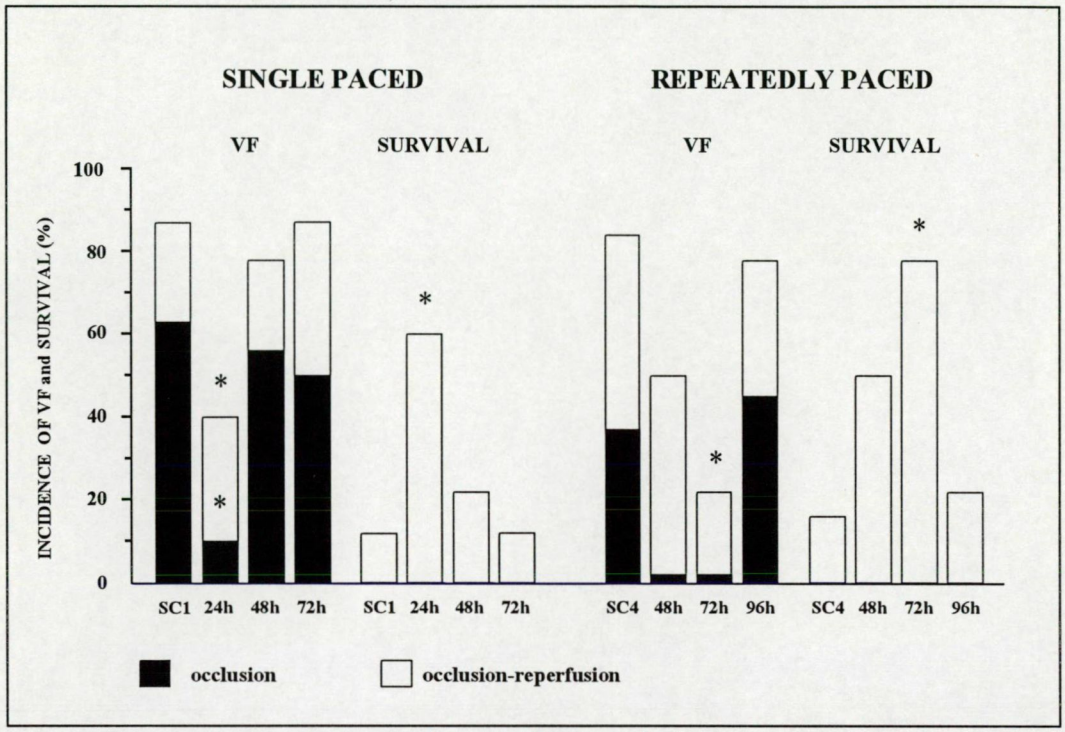

*Figure 6. The incidence of ventricular fibrillation (VF) and survival during occlusion and after reperfusion in sham control dogs (SC1, SC4) and in dogs subjected to a period of single pacing (4x5 min) 24, 48 and 72 h before coronary artery occlusion and in repeatedly paced dogs subjected to LAD occlusion 48, 72 and 96 h after the second pacing stimulus. (Data of SC1 and single paced groups taken from Ref 30). * $P<0.05$ vs. sham control.*

Since in the present study there was no significant difference between the two time-matched sham-control groups (96h, n=11 and 120h, n=10, Figure 4) we combined these animals in one group (SC4, n=21). In this combined sham-control group nearly all dogs exhibited severe ventricular arrhythmias following coronary artery occlusion. The number of ventricular premature beats (VPBs; 231±69) and the number of episodes of ventricular tachycardia (VTepisodes; 4.4±1.6) were high in these animals. VT occurred during the occlusion period in 14 out of 21 sham control dogs, 7 dogs fibrillated (33%) during the occlusion period and 10 out of the 14 remaining dogs which survived the occlusion fibrillated following reperfusion. Thus, only 4 out of 21 sham control dogs survived the combined occlusion-reperfusion insult (Figure 7).

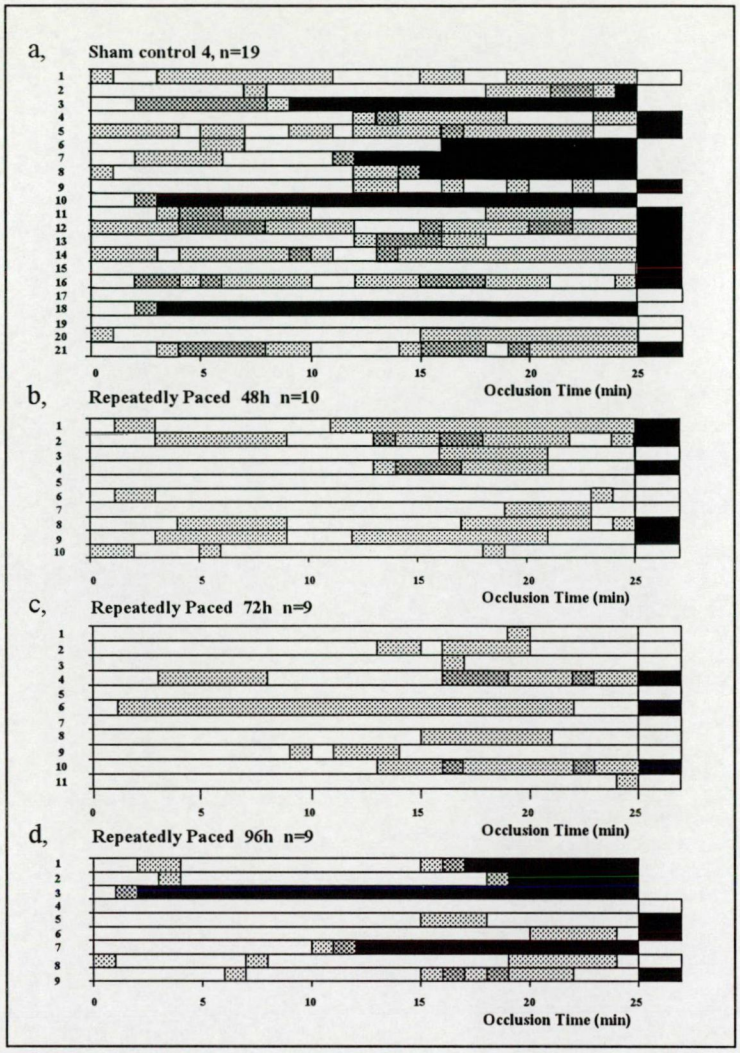

Figure 7. Distribution of ventricular arrhythmias: ventricular ectopic beats (VPBs, indicated as light grey), ventricular tachycardia (VT, indicated as dark grey), and ventricular fibrillation (VF, indicated as black) in sham operated control dogs (a, panel), and repeatedly paced dogs subjected to a 25 min occlusion of the LAD 48, 72 or 96 h later (b, c, d, panels).

All arrhythmia parameters were markedly suppressed in those dogs which were subjected to repeated pacing 48 and 72 h previously. For example, few ectopic beats (VPBs: 95±39; 100±47, respectively) occurred in these dogs; the incidence of VT was low (20% and 18%, respectively) and no dog fibrillated during the occlusion period in these two repeatedly paced groups. Repeated cardiac pacing significantly increased survival from the combined ischaemia-reperfusion insult 48 and 72 h later, 50% and 73% of these dogs survived, respectively (Figures 6 and 7).

When the time interval between the second pacing stimulus and the occlusion was extended to 96 hours the antiarrhythmic protection faded. Although the number of ectopic beats and VT episodes was still suppressed, 45% of the dogs fibrillated during the occlusion and only 2 out of 9 dogs survived the reperfusion.

3.1.4. Changes in the indices of ischaemia severity

The severity of myocardial ischaemia was expressed by changes in epicardial ST-segment elevation and in the degree of inhomogeneity of electrical activation within the ischaemic area. In the sham control group pronounced epicardial ST-segment elevation developed within 3 min of the occlusion, reached a maximum at 5 min and remaining stable during the rest of the occlusion period. In those dogs which were previously subjected to repeated pacing, the ischaemia-induced epicardial ST-segment elevation was significantly reduced; this was especially marked in those dogs which were subjected to repeated pacing 48 or 72 h previously (Figure 8). Changes in the degree of electrical inhomogeneity were similar to changes in epicardial ST-segment elevation. Thus, compared to the sham controls, in dogs paced repeatedly 48, 72 h and even 96 h before the occlusion the degree of inhomogeneity was significantly reduced over the entire occlusion period (Figure 9).

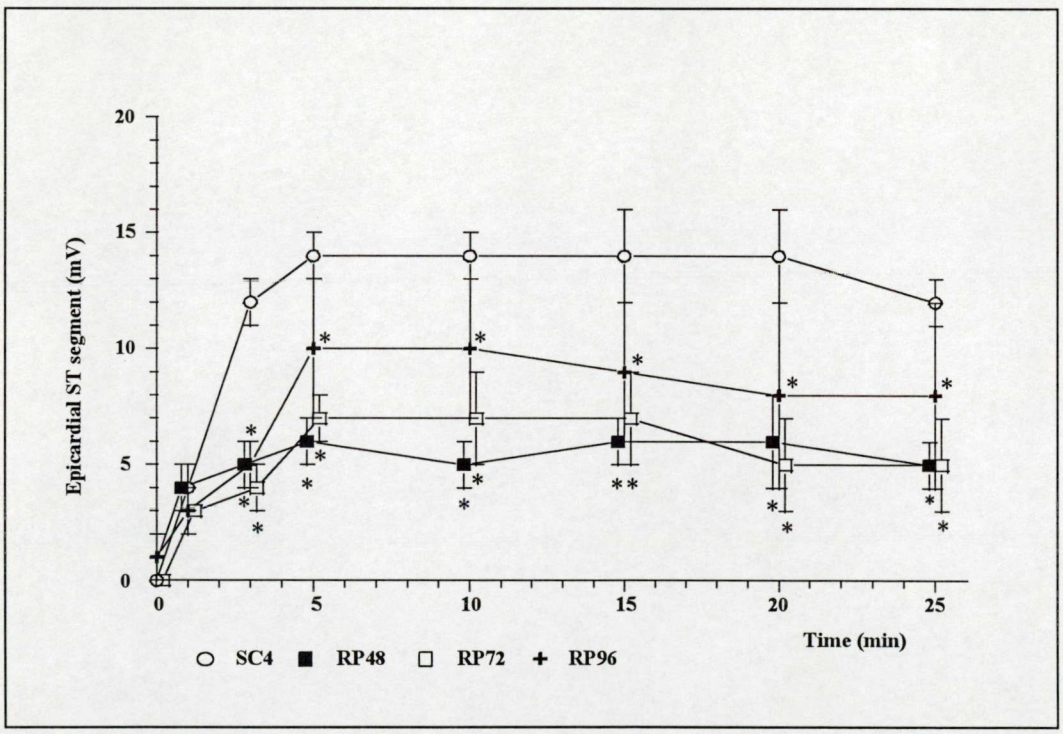

*Figure 8. Changes in epicardial ST segment elevation (mV) during 25 min occlusion of the left anterior descending coronary artery in sham control dogs (SC4) and in repeatedly paced dogs (RP48, RP72, RP96). *P<0.05 vs. sham controls.*

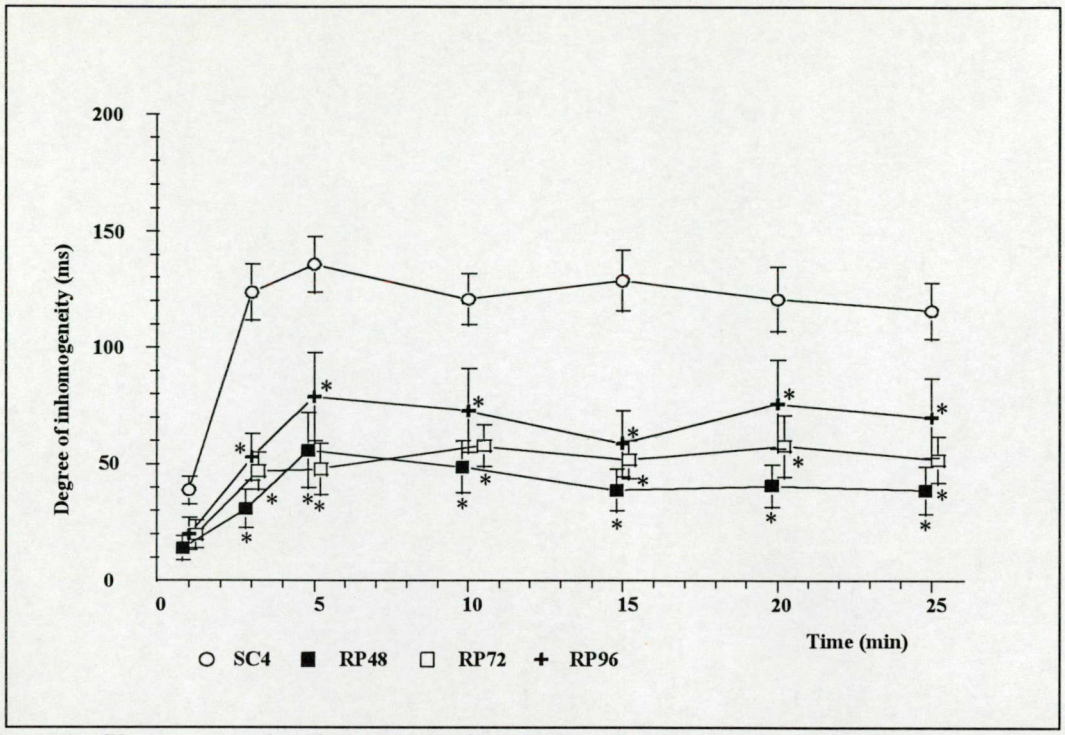

*Figure 9. Changes in the degree of inhomogeneity of electrical activation (ms) during 25 min occlusion of the left anterior descending coronary artery in sham control dogs (SC4) and in repeatedly paced dogs (RP48, RP72, RP96). *P<0.05 vs. sham controls.*

3.2. Evidence for the role of nitric oxide and prostacyclin in classical preconditioning

3.2.1. Haemodynamic effects of administration of meclofenamate and L-NAME

Intravenous administration of meclofenamate had no significant haemodynamic effects (Table 3). However, infusion of L-nitroarginine methylesther (L-NAME) into a small branch of the left anterior descending coronary artery significantly increased arterial blood pressure (SABP, DABP, MABP), left ventricular systolic pressure (LVSP) and significantly decreased heart rate (HR). There were no marked changes in left ventricular end-diastolic pressure, in positive or negative LVdP/dt.

Table 3.	Haemodynamic effects of meclofenamate and L-NAME			
	Pre-meclofenamate	change	Pre-L-NAME	change
SABP (mmHg)	121±8	6±2	125±8	16±3*
DABP (mmHg)	78±4	4±3	80±5	26±2*
MABP (mmHg)	92±5	5±2	95±5	22±2*
LVSP (mmHg)	125±7	5±2	129±7	18±3*
LVEDP (mmHg)	6±1	0	7±1	1±1
+dP/dt (mmHg s^{-1})	2746±162	118±78	2817±204	218±126
-dP/dt (mmHg s^{-1})	2560±114	192±66	2674±100	171±119
HR (beats min^{-1})	151±9	2±1	153±10	-8±2*

*Table 3. Haemodynamic changes induced by meclofenamate (2 mg kg^{-1}) and L-NAME (5 mg kg^{-1}) in chloralose-urethane anaesthetised dogs. * $P<0.05$ vs. baseline.*

3.2.2. The severity of ventricular arrhythmias during the preconditioning procedure induced by brief coronary artery occlusions

Occlusion of the LAD proximal to the first main diagonal branch in dogs (Figure 1) results in a 35-40% area at risk and leads to the occurrence of ventricular arrhythmias such as ventricular ectopic beats, tachycardia and even fibrillation. These arrhythmias appear within a few minutes after ligation of the coronary artery. When dogs are preconditioned by brief coronary artery occlusion usually 25 % of these dogs fibrillate either during the first preconditioning occlusion or immediately after reperfusion of the ischaemic myocardium (Figure 10). In the presence either of L-NAME or sodium meclofenamate given prior to preconditioning, the incidence of VF during the preconditioning procedure was increased to 30% or 45%, respectively. However, when both meclofenamate and L-NAME were given together prior to the first 5 min occlusion 78% of the dogs fibrillated during the preconditioning procedure (Figure 10).

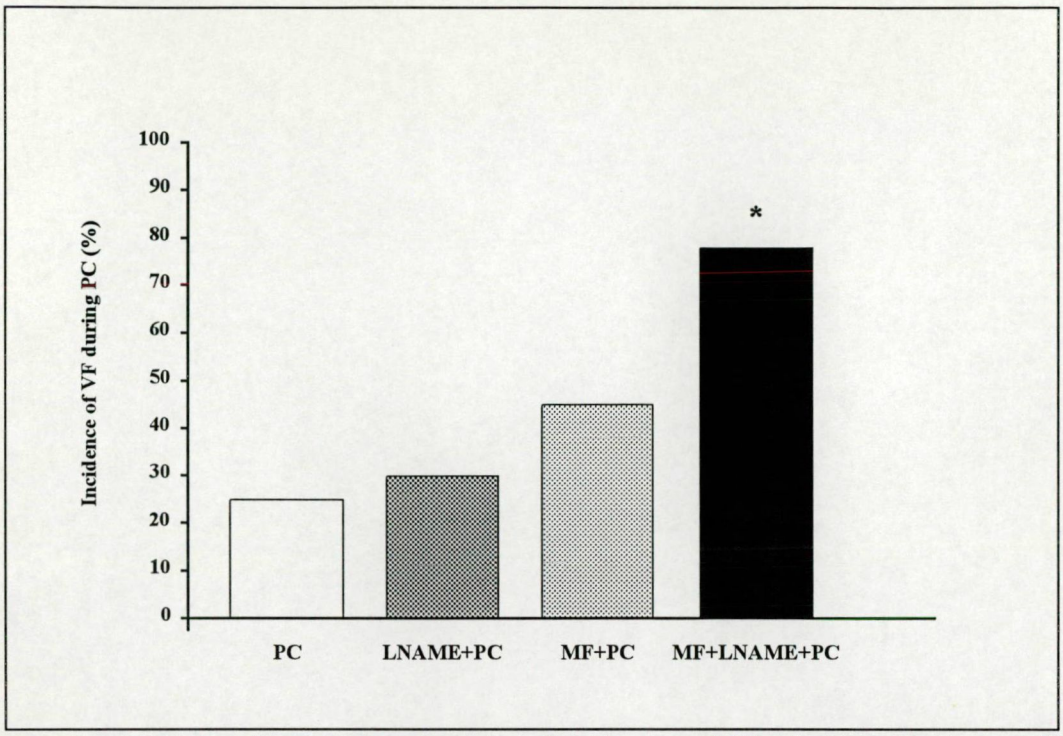

*Figure 10. The incidence of ventricular fibrillation (VF) during the preconditioning procedure (PC), in preconditioned dogs treated with L-NAME (L-NAME+PC) or sodium meclofenamate (MF+PC), and in dogs in which both meclofenamate and L-NAME were administered prior to the preconditioning procedure (MF+L-NAME+PC). * $P<0.05$ vs PC.*

3.2.3. The severity of ventricular arrhythmias during prolonged occlusion in preconditioned dogs treated with L-NAME and meclofenamate

Ischaemic preconditioning by brief coronary artery occlusions protects the heart against those arrhythmias which occur during the subsequent prolonged ischaemic insult (7). Thus, compared to the control group in preconditioned dogs the number of ventricular premature beats (VPBs, 72±21 vs. 439±72, $P<0.01$) and episodes of ventricular tachycardia (VT, 1.1±0.5 vs. 7.6±2.1, $P<0.01$) was significantly decreased during prolonged occlusion (Figure 11). Furthermore, preconditioning markedly reduced the incidences of the more malignant ventricular arrhythmias such as VT or VF both during occlusion and reperfusion. Thus, in the control group 42% of the dogs fibrillated during the occlusion period and those which survived the prolonged ischaemia fibrillated after reperfusion (Figure 12). In contrast, in the preconditioned group no dog fibrillated during occlusion, and only 11 out of 22 dogs fibrillated after reperfusion. Thus, 50% of the preconditioned dogs survived the combined occlusion-reperfusion insult (Figure 12).

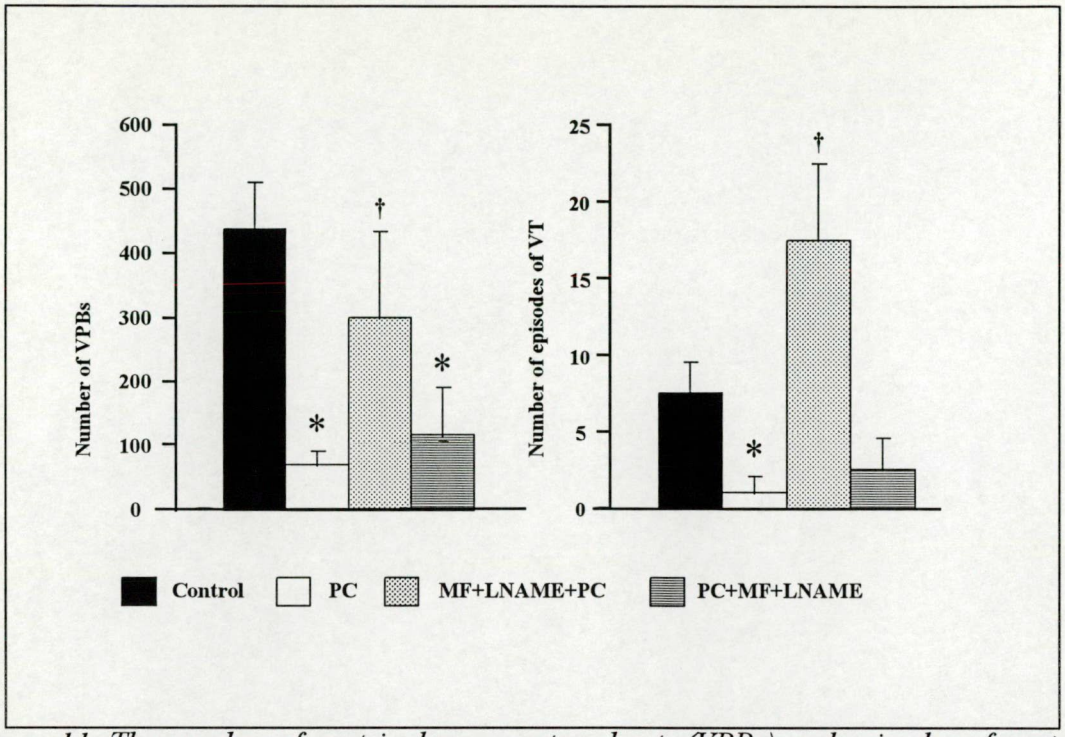

*Figure 11. The number of ventricular premature beats (VPBs) and episodes of ventricular tachycardia (VT) during a 25 min coronary artery occlusion in control, preconditioned (PC), and also those preconditioned dogs which were treated with MF and L-NAME before or after the preconditioning procedure. * $P<0.05$ vs. control group. † $P<0.05$ vs. PC group.*

When both meclofenamate and L-NAME were given together *before* the preconditioning procedure the number of VPBs and the number of episodes of VT were significantly increased (72±21 vs. 301±134, 1.1±0.5 vs. 17.5±4.5, $P<0.01$) compared to the dogs preconditioned without administration of drugs (Figure 11). However, when meclofenamate and L-NAME were administered *after* preconditioning, but prior to the prolonged occlusion, the number of VPBs and episodes of VT during sustained ischaemia was still markedly reduced compared to the controls (118±74 vs. 439±72, $P<0.05$, and 2.6±2.2 vs. 7.6±2.1, Figure 11). Dual blockade of cyclooxygenase and nitric oxide synthase enzymes before ischaemic preconditioning resulted in high incidences of VT (100%) and VF (50%) during occlusion and no dog survived the combined occlusion-reperfusion insult in this group (Figure 12). In dogs treated with both meclofenamate and L-NAME after preconditioning, but before sustained ischaemia, the number of VPBs and episodes of VT was still markedly suppressed similar to that seen in preconditioned dogs, but the incidences of VT and VF during occlusion and after reperfusion were as the same as in the control group (Figure 12).

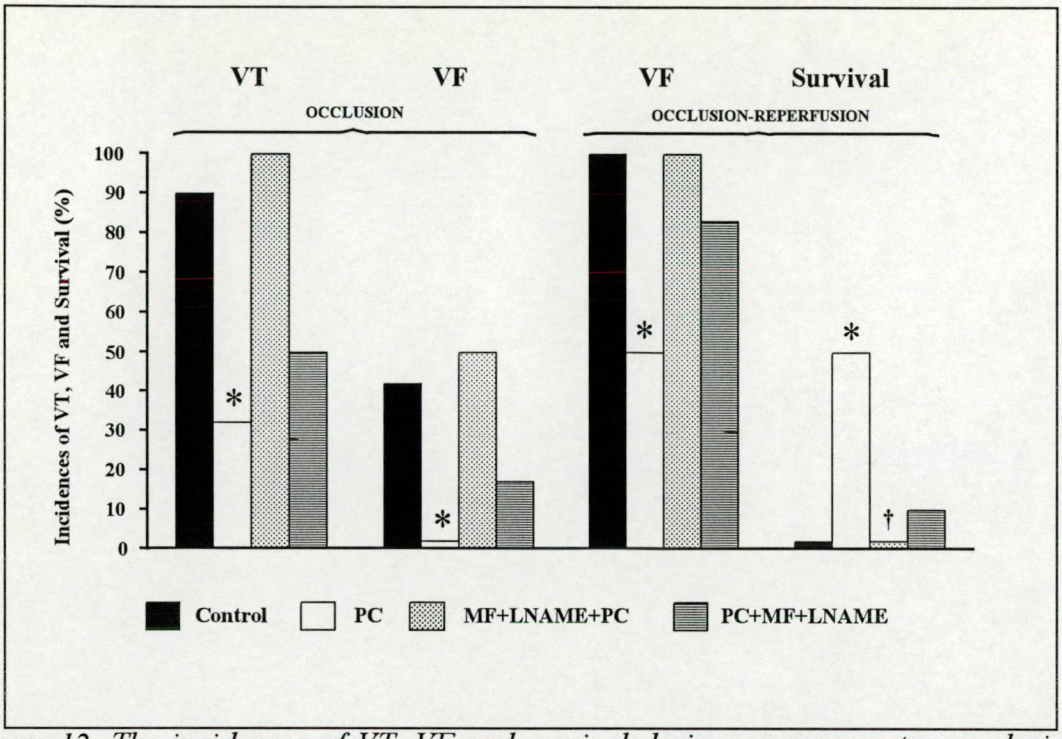

*Figure 12. The incidences of VT, VF and survival during coronary artery occlusion and reperfusion in control, preconditioned dogs (PC) and in those preconditioned groups in which the animals were treated with MF and L-NAME either before or after the preconditioning procedure. * $P<0.05$ vs. control group. † $P<0.05$ vs. PC group.*

3.2.4. The effect of meclofenamate and L-NAME on the severity of myocardial ischaemia induced by coronary artery occlusion

Compared to the control group preconditioning markedly reduced the severity of ischaemia as shown by a significant decrease in epicardial ST-segment elevation (Figure 13) and in the degree of inhomogeneity of electrical activation measured within the ischaemic region (Figure 14). The protective effect of preconditioning on the degree of inhomogeneity disappeared in the presence of meclofenamate and L-NAME, whereas the protection was still present regarding changes in the epicardial ST-segment elevation, particularly when the drugs were given after preconditioning but prior to the prolonged occlusion of the LAD (Figure 13).

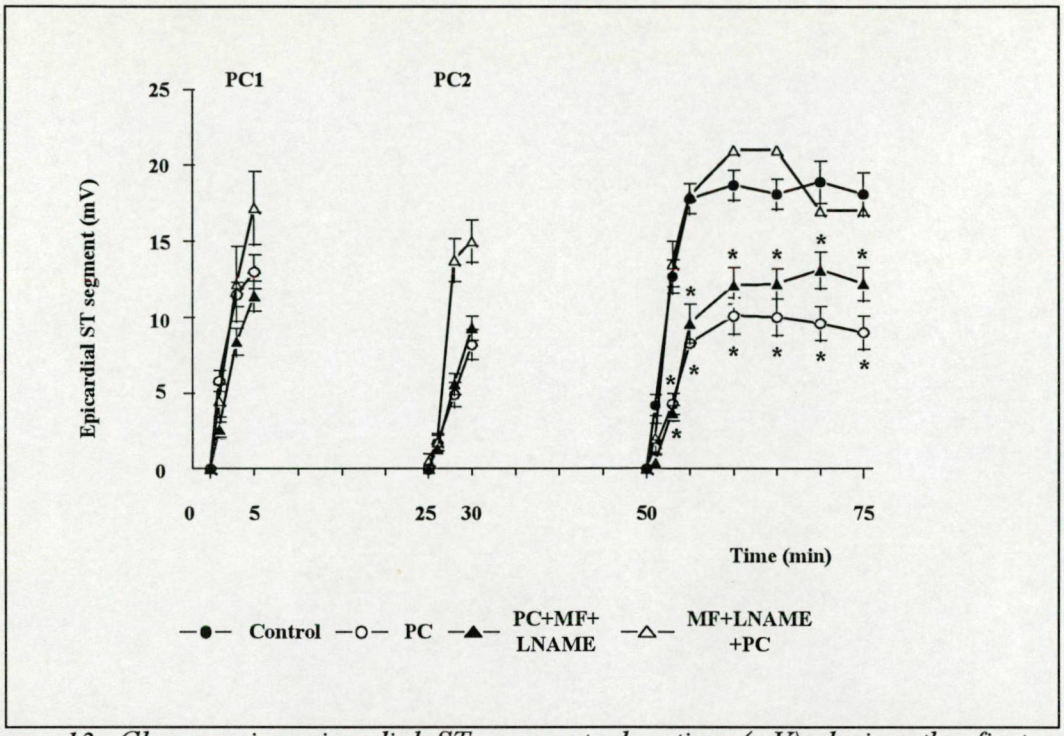

*Figure 13. Changes in epicardial ST segment elevation (mV) during the first and the second episodes of preconditioning occlusion, and over a 25 min occlusion of the LAD in control, preconditioned dogs (PC), and in dogs in which sodium meclofenamate and L-NAME were given either prior to the preconditioning or prior to prolonged occlusion. * P<0.05 vs. control group.*

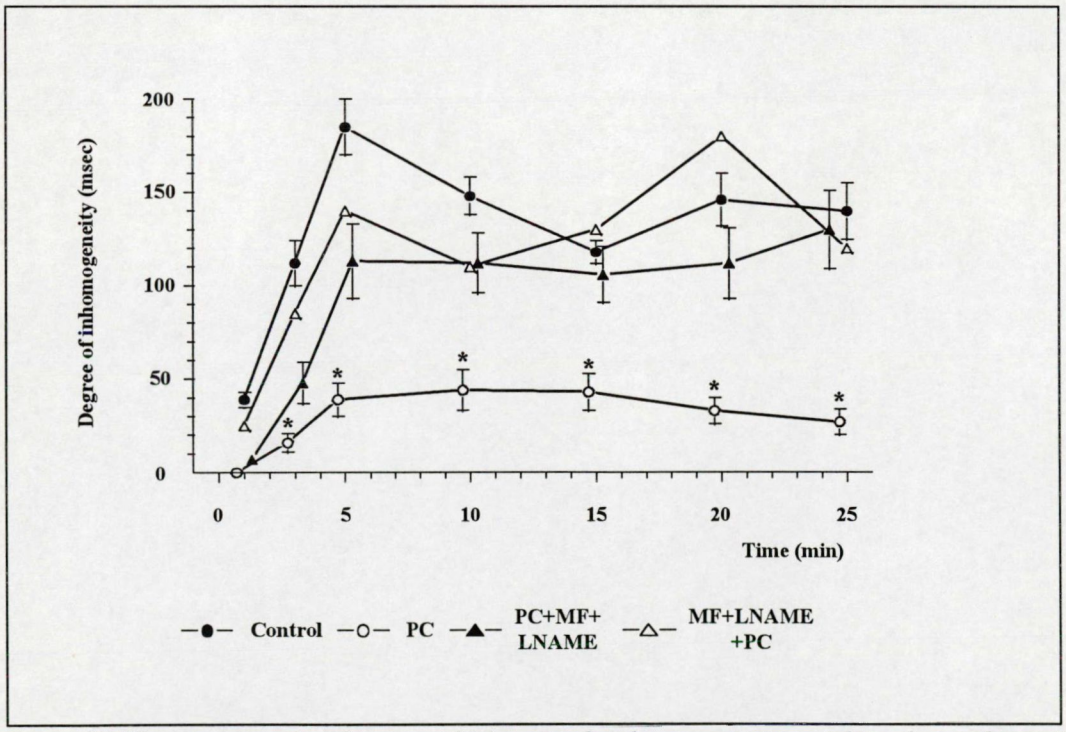

*Figure 14. Changes in the degree of electrical inhomogeneity within the ischaemic area during a 25 min occlusion of the LAD in control dogs, in preconditioned dogs (PC), and in dogs in which meclofenamate and L-NAME were given either prior to or after preconditioning. * P<0.05 vs. control group.*

3.3. Evidence for the role of nitric oxide in delayed protection induced by right ventricular pacing

3.3.1. Haemodynamic effects of administration of aminoguanidine in paced and unpaced dogs

Aminoguanidine in a dose of 50 mg kg^{-1} given 30 minutes prior to the pacing stimulus, or just prior to the coronary artery occlusion, significantly increased systolic, diastolic and mean arterial blood pressure and decreased heart rate. Data are summarised in Table 4. Most of these changes returned to the baseline before the commencement of the coronary artery occlusion.

Table 4. Haemodynamic effects of aminoguanidine (50mg kg^{-1}) in dogs

			Time after injection (min)			
	Groups	Baseline	1	5	15	30
SABP (mmHg)	P+AG	139±6	141±9	157±8^{b}	151±7	147±9
	AG+occl	136±7	140±7	176±5^{c}	161.5±7^{c}	156±10^{b}
	AG+P	180±6	187±8	192±7^{b}	188±6^{a}	188±7
DABP (mmHg)	P+AG	92±5	91±7	113±5^{c}	102±5	99±6
	AG+occl	95±3	99±4	129±5.5^{c}	114±3^{c}	106±5^{b}
	AG+P	138±3	144±5	151±3^{c}	145±4^{c}	145±5^{a}
MABP (mmHg)	P+AG	107±5	107±7	128±6^{c}	118.5±6	115±7
	AG+occl	109±4	113±5	145±6^{c}	130±4^{c}	123±6^{b}
	AG+P	152±4	158±6	165±4^{c}	159±4^{b}	159±5^{a}
HR (beats min^{-1})	P+AG	146±9	139±8^{a}	134±7^{b}	143±9	143±8
	AG+occl	144±5	144±6	132±7^{c}	145±2	148±8
	AG+P	152±4	158±6	165±4^{c}	159±4^{b}	159±5^{a}

Table 4. Changes in the haemodynamic parameters following aminoguanidine given either 30 min prior to the pacing stimulus (AG+P) or just prior to the coronary artery occlusion in paced (P+AG) and in unpaced dogs (AG+occl). SABP: systolic arterial blood pressure (mmHg), DABP: diastolic arterial blood pressure (mmHg), MABP: mean arterial blood pressure (mmHg), HR: heart rate (beats min^{-1}). a: $P<0.05$ vs. baseline, b: $P<0.01$ vs. baseline, c: $P<0.001$ vs. baseline.

3.3.2. Delayed protection by right ventricular pacing against ischaemia-reperfusion induced arrhythmias 24 h later; effects of aminoguanidine

Rapid cardiac pacing results in pronounced protection against those ventricular arrhythmias which occur during a 25 min occlusion of the left anterior descending coronary artery 24 h later. Thus, compared to the sham controls there were significant reductions in the number of VPBs (70±28 vs. 330±110, $P<0.05$) and in the number of episodes of VT (2.2±1.9 vs. 6.4±3.6) in dogs paced 24 h previously (Figure 15). Right ventricular pacing also protected the

heart against life-threatening ventricular arrhythmias which occurred either during occlusion or after reperfusion. Thus, compared to the sham control group in which almost 60% of the dogs fibrillated during occlusion only 15% of the dogs which were subjected to right ventricular pacing 24 h earlier fibrillated during this time. In the control group, 3 out of those dogs which survived the LAD occlusion fibrillated after reperfusion. Thus, in this group 2 out of 12 dogs survived the combined occlusion-reperfusion insult. In contrast 62% of the dogs survived the combined occlusion-reperfusion insult which were paced 24 h before the occlusion (Figure 16).

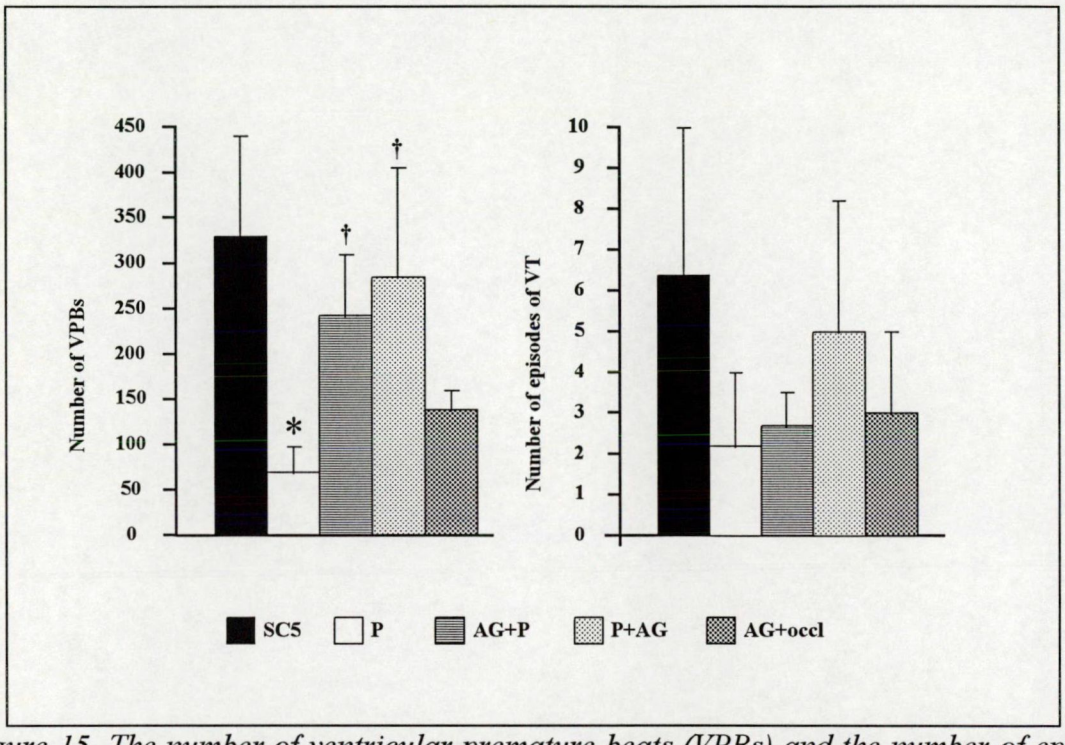

*Figure 15. The number of ventricular premature beats (VPBs) and the number of episodes of ventricular tachycardia (VT) during coronary artery occlusion in sham control dogs (SC5), in dogs paced 24 h previously without aminoguanidine (P) and in paced dogs in which AG was given either 30 minutes prior to the pacing stimulus (AG+P) or prior to the occlusion of the LAD (P+AG) as well as in unpaced dogs subjected to a 25 min occlusion of the LAD (AG+occl). * $P<0.05$ vs. sham control group. † $P<0.05$ vs paced group.*

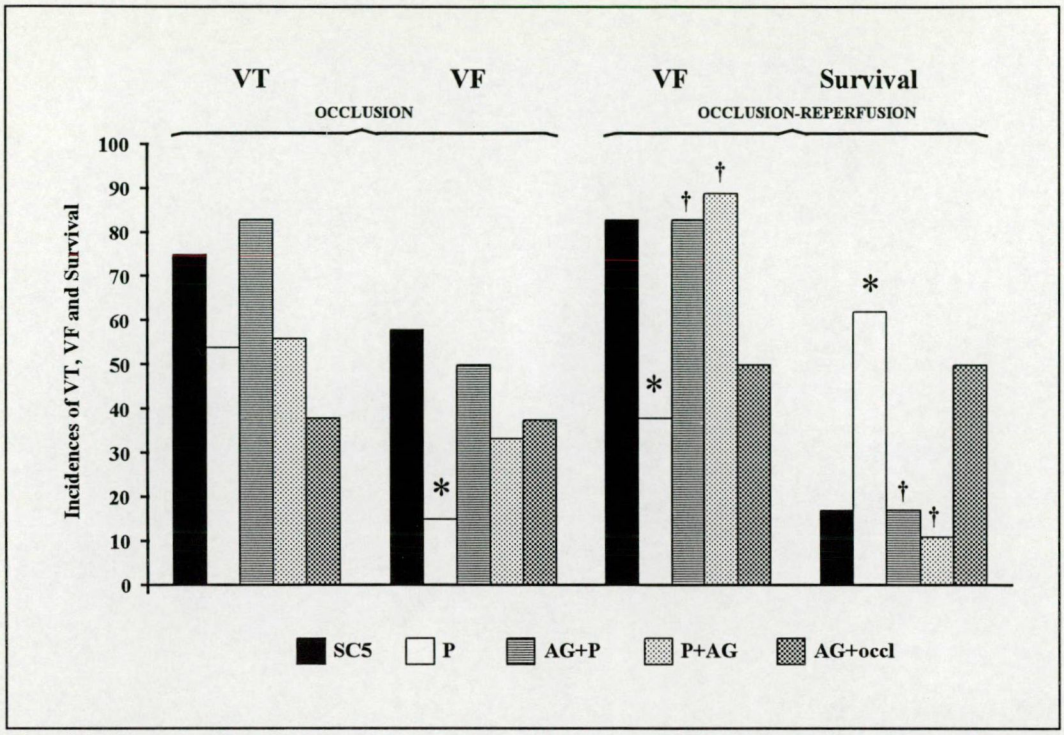

*Figure 16. The incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and survival in sham control dogs (SC5), in dogs paced 24 h before occlusion (P) and in paced dogs in which AG was given 30 minutes either prior to the pacing stimulus (AG+P) or prior to the occlusion of the LAD (P+AG) as well as in unpaced dogs subjected to 25 min occlusion of the LAD (AG+occl). * $P<0.05$ vs. sham control group. † $P<0.05$ vs. paced group.*

Administration of aminoguanidine markedly attenuated the antiarrhythmic protection resulted from cardiac pacing. Aminoguanidine given either prior to the pacing stimulus or prior to the coronary artery occlusion resulted in similar increase in the severity of ventricular arrhythmias. The number of VPBs was as high as in the control group (243±67 and 285±120 in paced dogs given AG prior to pacing and prior to occlusion, respectively, Figure 15). The protection against VT and VF during occlusion was lost in the AG treated animals, and only 17% of the dogs given the drug prior to pacing and 11% of the animals treated with AG before occlusion survived the combined ischaemic-reperfusion insult (Figure 16).

Interestingly, AG given to unpaced dogs 30 minutes prior to coronary artery occlusion resulted in a slight reduction, rather than an increase, in the severity of ventricular arrhythmias during ischaemia (Figure 15 and 16).

3.3.3. Effect of cardiac pacing on epicardial ST-segment elevation and on the degree of inhomogeneity of electrical activation; effects of aminoguanidine

These are illustrated in Figures 17 and 18. Right ventricular pacing 24 h prior to coronary artery occlusion significantly reduced the severity of myocardial ischaemia compared to the control group as indicated by marked reduction in epicardial ST segment elevation and in the degree of electrical inhomogeneity. This protection was reversed by aminoguanidine when it was administered in paced dogs either prior to the pacing stimulus or prior to the coronary artery occlusion. Aminoguanidine itself did not modify these indices of ischaemia severity in unpaced dogs.

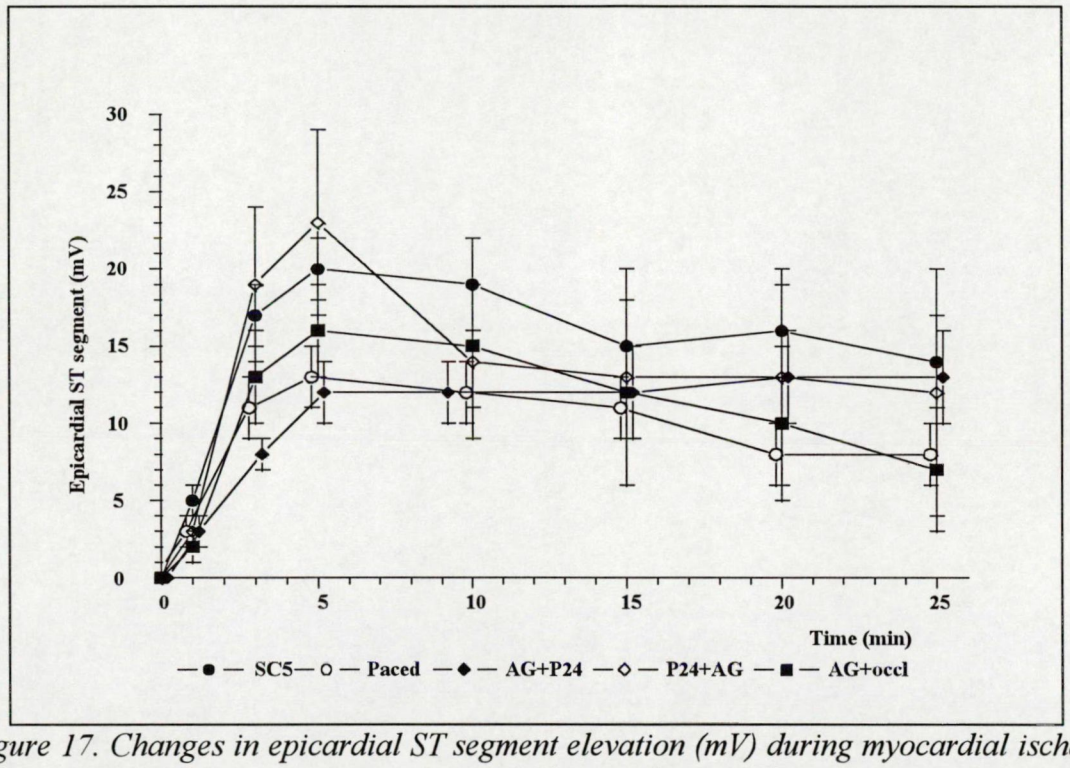

Figure 17. Changes in epicardial ST segment elevation (mV) during myocardial ischaemia in sham control (SC5), in dogs paced 24 h before occlusion (P), and in paced dogs treated with aminoguanidine 30 minutes either prior to the pacing stimulus (AG+P) or prior to the coronary artery occlusion (P+AG), as well as in unpaced dogs subjected to 25 min occlusion of the LAD (AG+occl).

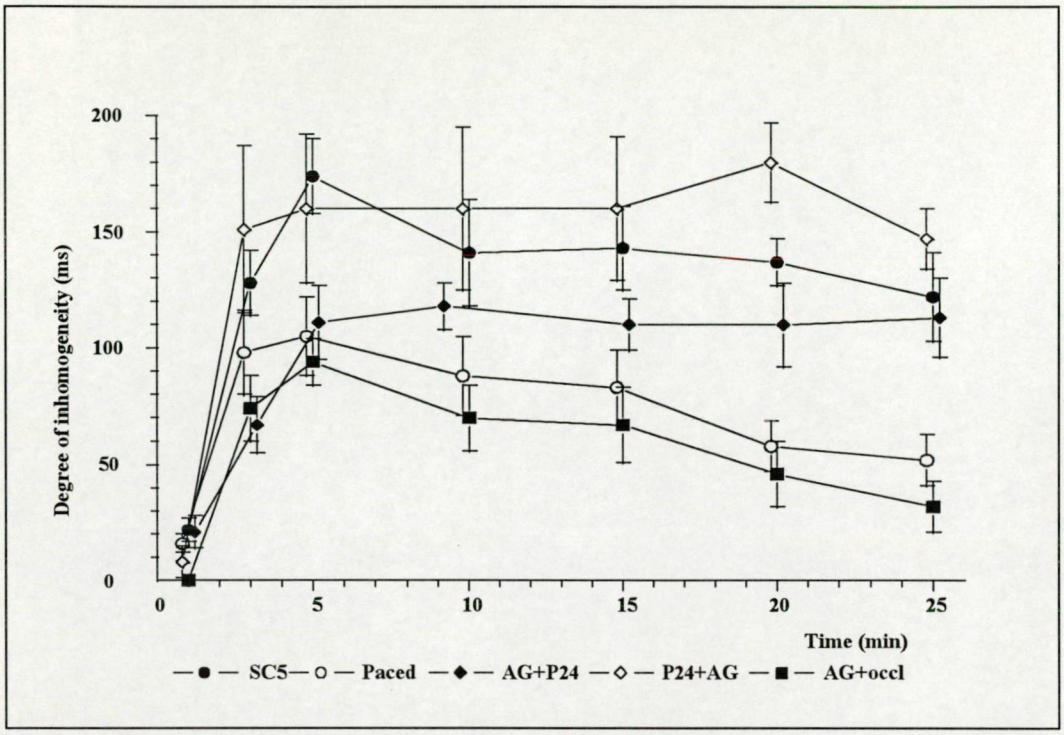

Figure 18. Changes in the degree of inhomogeneity of electrical activation during 25 min occlusion of the LAD in sham control (SC5),in dogs paced 24 h before occlusion (P), and in paced dogs treated with aminoguanidine 30 min either prior to pacing (AG+P) or prior to occlusion (P+AG), as well as in unpaced dogs (AG+occl).

3.4. Evidence for the role of nitric oxide in delayed protection induced by repeated pacing; effect of AEST on ventricular arrhythmias and on severity of myocardial ischaemia

There was no changes in haemodynamic parameters during infusion of AEST (data not shown).

As it was demonstrated in Figures 6 and 7 repeated cardiac pacing prolonged the protection against ischaemia and reperfusion-induced ventricular arrhythmias. Thus, compared to the sham controls (SC3), 72 h after the second pacing stimulus the number of VPBs (101±49 vs. 229±94, Figure 19) and the number of episodes of VT (1.6±1.5 vs. 3.9±2.1, Figure 19) were decreased. This protection against VPBs and VT episodes was largely attenuated when AEST, a reasonably selective inhibitor of the iNOS, was given prior to coronary artery occlusion. In these dogs the number of VPBs (237±98) and VT episodes (5.6±2.7) was markedly increased (Figure 19).

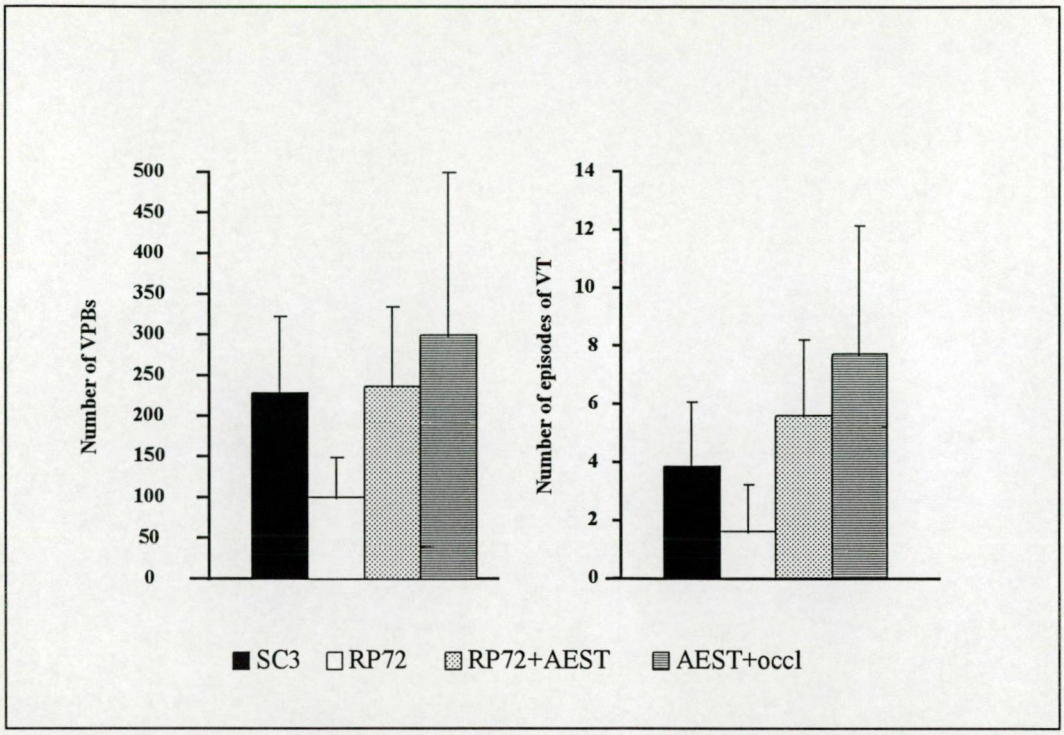

Figure 19. The number of ventricular premature beats (VPBs) and the number of episodes of ventricular tachycardia (VT) during coronary artery occlusion in sham control dogs (SC3), in dogs subjected to repeated pacing 72 h previously (RP72), in repeatedly paced dogs given AEST 90 minutes prior to the pacing stimulus (RP72+AEST) and in unpaced dogs treated with AEST prior to occlusion of the LAD (AEST+occl).

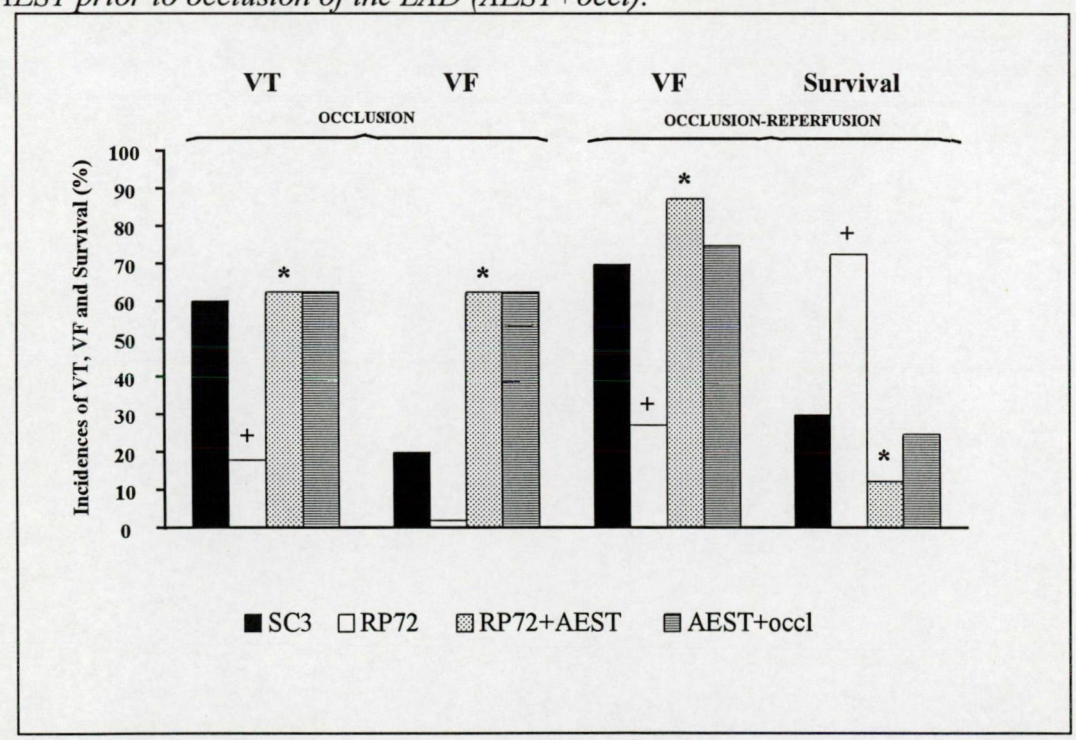

*Figure 20. The incidences of ventricular tachycardia (VT) and ventricular fibrillation (VF) and survival from the combined ischaemia-reperfusion insult in sham control dogs (SC3), in dogs subjected to repeated pacing 72h previously (RP72), in dogs repeatedly paced and given AEST 90 min prior to the pacing stimulus (RP72+AEST), and in unpaced dogs treated with AEST prior to occlusion of the LAD (AEST+occl). *: $P<0.05$ vs. RP72+AEST, +: $P<0.05$ vs. SC3.*

Similarly, compared to the control group, repeated pacing significantly reduced the incidence of VT (18% vs. 60%, $P<0.05$) during occlusion and the incidence of VF (27% vs. 70%, $P<0.05$) after reperfusion (Figure 20). Thus, survival from the combined occlusion-reperfusion insult was 30% in the sham controls and 73% in dogs paced repeatedly. AEST given prior to coronary artery occlusion in paced dogs increased the incidences of VT (to 63%), and VF (to 63%) during occlusion and also following reperfusion (88%). Thus, only one dog out of 8 survived in this group. AEST itself did not significantly modify the severity of ventricular arrhythmias (Figures 19 and 20).

Repeated cardiac pacing significantly reduced the severity of myocardial ischaemia compared to the sham control group as shown by the marked decrease in epicardial ST-segment elevation and in the degree of inhomogeneity. Administration of AEST prior to occlusion of the LAD in repeatedly paced dogs abolished this protective effect; ie. there was a rapid development in epicardial ST-segment elevation and in the degree of inhomogeneity. These changes were similar to that observed in the sham controls (Figure 21).

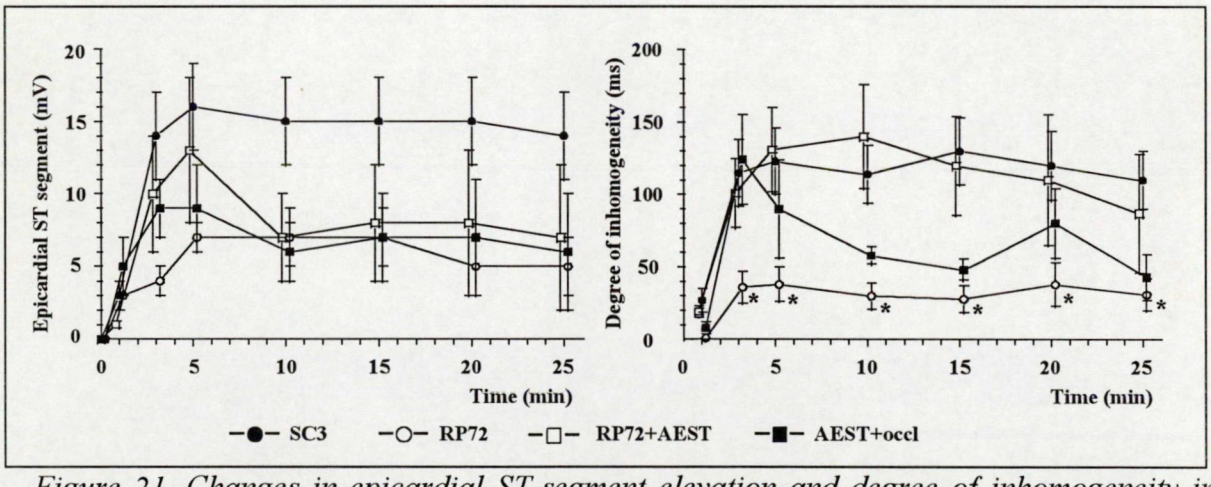

*Figure 21. Changes in epicardial ST-segment elevation and degree of inhomogeneity in sham control dogs (SC3), in dogs paced repeatedly (RP72), in repeatedly paced dogs treated with AEST (RP72+AEST) and in unpaced dogs given AEST prior to occlusion (AEST+occl). * $P<0.05$.*

3.5. Evidence that the inducible form of nitric oxide synthase plays a role in the delayed cardioprotection

3.5.1. Electromobility shift assay for the measurement of activation of nuclear factor κ-B

Activation of nuclear factor κ-B (NFκB) was analyzed by electromobility shift assay, a method used to detect the phosphorylated transcription factor in the nucleus by means of radioactive labelled specific oligonucleotide. The active transcription factor constitutes a homodimer or heterodimer from two different subunits (p65 and p50).

There was a small constitutive activity of the p65, but not of the p50, subunit in the rabbit and dog sham controls. In rabbits 10 min after ischaemic preconditioning both p50 and p65 subunits of the NFκB were already activated (approximately twice more compared to the sham control). Interestingly, NFκB starts to lose its activation 1 h after ischaemic preconditioning. At this time the p65 subunit was still activated and this activation was nearly completely lost after 3 h recovery (Figure 22). Although in rabbits slight activation was still apparent in p65 subunit 1 or 3 h following the preconditioning stimulus, the activation of the p50 subunit was markedly attenuated by that time.

Preconditioning induced by rapid cardiac pacing in dogs resulted in a very strong activation of the p65 subunit (approximately four times more than in the controls, Figure 22). Compared to the rabbits there was a shift in the time course of the activation of p65; the most pronounced activation of the factor was seen 1 h after cardiac pacing, a time when the antiarrhythmic protection has already faded. In dogs neither the p65 nor the p50 subunit of NFκB was activated 15 min or 24 h after pacing (Figure 22).

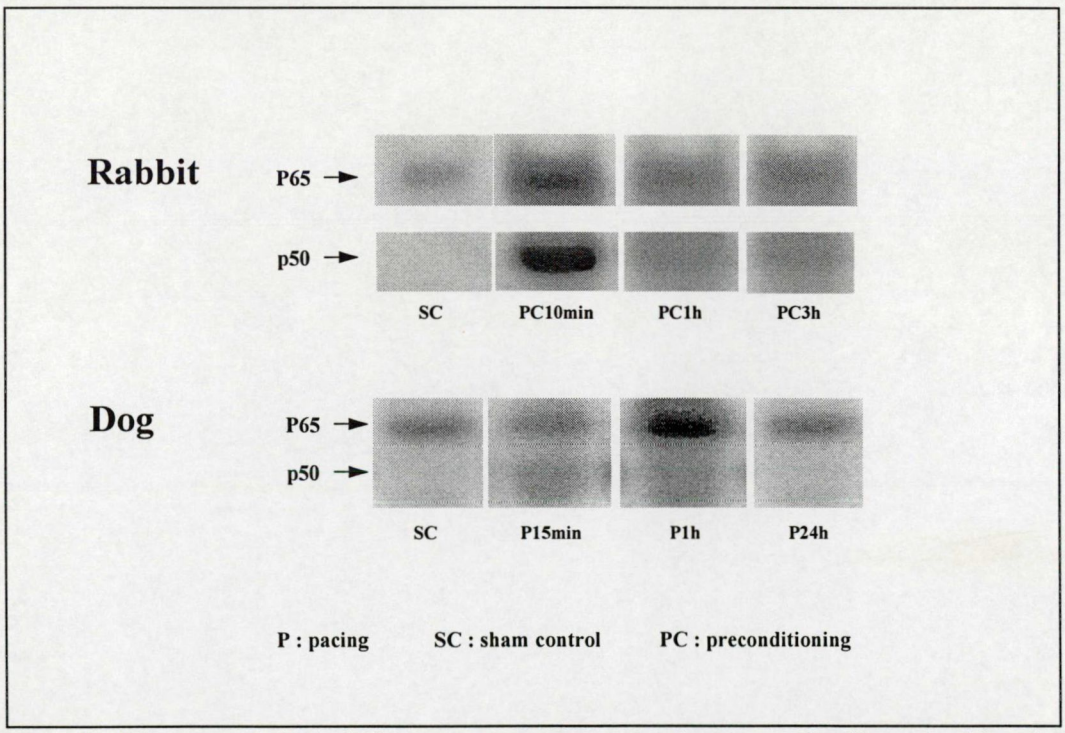

Figure 22. Electromobility shift assay to measure activation of p65 and p50 subunits in dogs paced 15 min, 1 h or 24 h previously and in rabbits preconditioned 10 min, 1 h and 3 h previously, and in the sham controls.

4. Discussion

4.1. Time course of delayed cardioprotection induced by preconditioning

It is well established that preconditioning induced either by short coronary artery occlusions or rapid cardiac pacing produces both early and delayed protection against severe consequences of myocardial ischaemia such as myocardial infarction and life-threatening ventricular arrhythmias. The degree of both these phases of the protection depends on the strength of preconditioning (i.e. the duration and the number of the preconditioning stimulus). For example, in rabbits four episodes of total coronary artery occlusion (total 20 min severe ischaemia) protects the heart against ischaemic damage which lasts for 72 h (44). In the same species 10 episodes of 5 min cardiac pacing, which probably does not result in as severe ischaemia as coronary artery occlusion, results in cardioprotection for 48 hours (35). In dogs four 5 min episodes of cardiac pacing through the right ventricle protects the heart against ventricular arrhythmias resulting from a subsequent coronary artery occlusion and reperfusion. This protection is apparent 24 h after pacing but fades by 48 h and is lost 72 h later (30). However, our experiments demonstrated if we repeat the pacing stimulus at a time when the antiarrhythmic effect of the previous pacing was virtually lost (i.e. at 48 h) the protection against these severe ventricular arrhythmias can be extended for a longer period. Thus, 48 h and 72 h after the second pacing stimulus the antiarrhythmic protection is still present (70). This protection starts to disappear 96 h after repeated pacing (70).

Prolonged delayed protection against myocardial infarction could be induced by repeated administration of 2-chloro-N6-cyclopenthyl-adenosine (CCPA), an A_1 adenosine receptor agonist (71). Given this drug at 48 h intervals for 10 days significantly reduced the extent of necrosis 48 h after the last bolus injection of the drug (71). Although this study did not attempt to examine the time course of the protection it can be assumed that this protective effect induced by repeated administration of CCPA lasts longer. However, as Hashimi and colleagues have pointed out chronic administration of an A_1 agonist could result in tachyphylaxis and loss of protection perhaps by downregulation or desensitization of A_1 receptors (72).

4.2. Possible mechanisms involved in the early phase of protection

Although the precise mechanisms of ischaemic preconditioning are not well established there is great deal of evidence that endogenous protective substances, released either from cardiac myocytes or vascular endothelial cells, or both, contribute to an adaptation associated with ischaemic preconditioning (47, 73, 74). It seems very likely that the early cardioprotection against ventricular arrhythmias in the canine heart induced either by short coronary artery occlusions or by cardiac pacing involves similar trigger mechanisms and mediators such as bradykinin, nitric oxide and prostacyclin (6, 56, 57, 60, 63-65, 75).

Previous studies have demonstrated that both prostacyclin and nitric oxide play an important role in the mechanism of protection against ventricular arrhythmias resulting from coronary artery occlusion and reperfusion (6, 47, 73, 74). Inhibition of cyclooxygenase and nitric oxide synthase enzymes with meclofenamate and with L-NAME, respectively, markedly attenuates the antiarrhythmic effects of ischaemic preconditioning in anaesthetised dogs (6, 63). When these inhibitors were administered simultaneously prior to preconditioning the protection against ventricular arrhythmias was completely abolished (76). Moreover it was difficult even to precondition the dogs when these endogenous pathways were simultaneously blocked (Figure 10).

There is substantial evidence that bradykinin triggers the generation of prostacyclin and nitric oxide (61, 62, 65). Local intracoronary infusion of bradykinin before coronary artery occlusion in dogs results in a significant reduction in the severity of myocardial ischaemia (reduced ST-segment elevation and degree of inhomogeneity of electrical activation) and suppresses ventricular arrhythmias (56). Furthermore, the protective effect of preconditioning against ventricular arrhythmias is abolished by icatibant, indicating the involvement of bradykinin and the subsequent activation of bradykinin B_2 receptors as a consequence of preconditioning (57, 77, 78). Bradykinin also plays a trigger role in the protective effect of preconditioning against ischaemic damage. Intravenous infusion of bradykinin significantly reduces the extension of myocardial infarction and this is prevented by icatibant (58, 59). Bradykinin has been found to inhibit noradrenaline release from nerve endings by activating B_1 receptors; this may contribute to the suppression of phase 1b arrhythmias during occlusion (79).

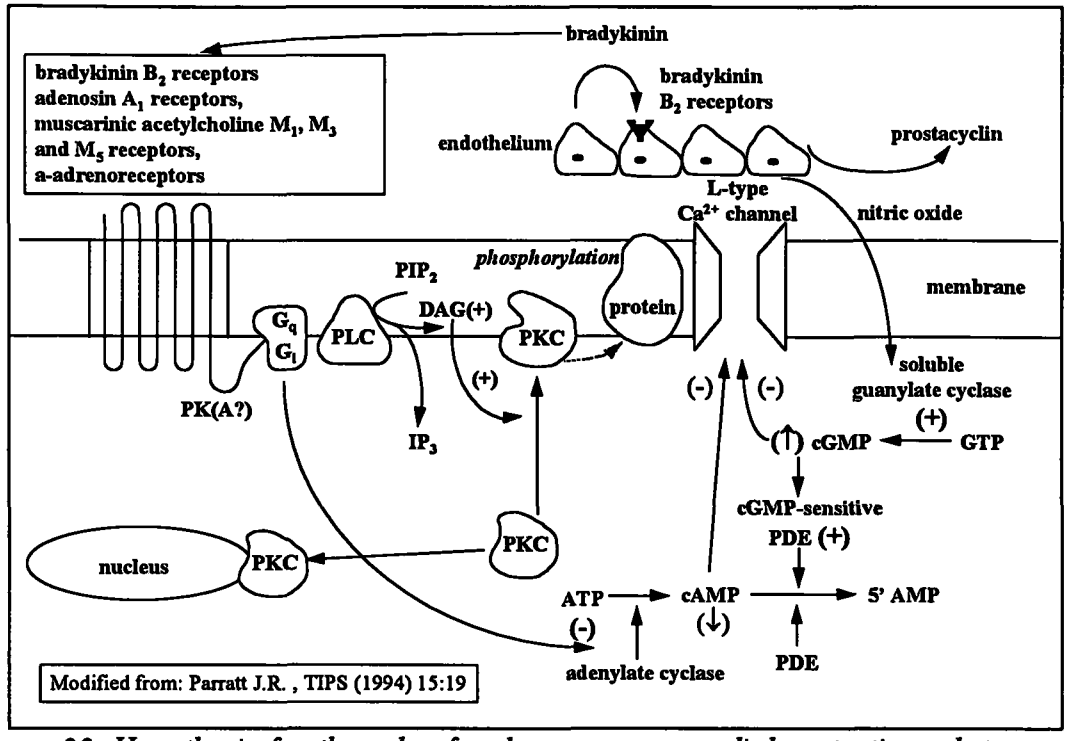

Figure 23. Hypothesis for the role of endogenous myocardial protective substances in the mechanism of the early antiarrhythmic protection induced by preconditioning in the canine myocardium.

On the basis of these findings described above, a possible hypothesis for the mechanism of the antiarrhythmic protection might be that bradykinin generated during the early phase of ischaemia activates the B_2 receptors in the endothelial cells and triggers the generation of prostacyclin and nitric oxide from the endothelium (and possibly also from cardiac myocytes). One mechanism for this protection might be the elevation of cGMP level as a result of activated soluble guanylate cyclase by nitric oxide. This would depress myocardial contractility and reduce oxygen demand during ischaemia, perhaps by limiting myocardial cAMP level by stimulation of the cGMP dependent phosphodiestherase enzyme. The increased level of cGMP can also inhibit L-type Ca^{2+} channels in the cardiac myocytes (Figure 23).

There is also some evidence that bradykinin, like adenosine, can translocate protein kinase C (PKC) from the cytosol into the cell membrane (and nuclear membrane) where it then phosphorylates membrane protein perhaps linked to ATP-dependent potassium channels. In theory opening of these channels should be cardioprotective by shortening the action potential and reducing the entry of calcium, a key player both in ischaemic damage and generation of arrhythmias.

4.3. Possible mechanisms involved in the delayed phase of protection induced by preconditioning

The mechanisms of delayed cardioprotection are less well established. The first study which suggested that nitric oxide may play a role in this delayed phase of protection was one in which dexamethasone given prior to cardiac pacing prevented the delayed antiarrhythmic protection in dogs when the coronary artery was occluded 24h later (41). This study indicated that either the inducible form of nitric oxide synthase or cyclooxygenase-2, or perhaps both, are involved in this protection. A study by Wu and colleagues supports the concept of a role for NO generated through activation of iNOS in delayed protection against ventricular arrhythmias. They have shown that dexamethasone markedly attenuated the endotoxin-induced antiarrhythmic protection in perfused rat hearts subjected to global ischaemia 24h later (80).

Our experiments, in which selective inhibitors of iNOS (aminoguanidine or AEST) were used, also support the hypothesis that delayed antiarrhythmic protection might be mediated, at least in part, by nitric oxide most probably generated by iNOS. The protection induced by cardiac pacing against ventricular arrhytmias was attenuated in dogs which had been subjected to one or two periods of pacing by aminoguanidine (81) or AEST (70). Although the protection is most probably due to induction or enhanced activation of iNOS we cannot exclude the involvement of the constitutive form of nitric oxide synthase in this phase of the protection. This is likely since in our studies administration of aminoguanidine given either prior to pacing or coronary artery occlusion resulted in an immediate elevation in arterial blood pressure presumably due to inhibition of cNOS (81). There is some evidence that during exercise excess generation of nitric oxide results from the upregulation of cNOS (82). Imagawa and his co-workers have shown that in anaesthetised rabbits nitric oxide plays a role as a mediator of the delayed preconditioning induced by brief coronary artery occlusions (83). The delayed protection against ischaemic damage was abolished by the prior administration of aminoguanidine. Bolli and his colleagues have described the late effect of ischaemic preconditioning against myocardial stunning in pigs (45) and later in rabbits (84). The authors suggest that NO triggers, and also mediates, the late effect of ischaemic preconditioning against myocardial stunning.

Long-term protection of the myocardium can also be evoked by a single dose of monophosphoryl lipid A (MLA), a nontoxic derivative of Salmonella thyphimurium endotoxin. MLA provides protection against necrosis, contractile dysfunction, ventricular arrhythmias and ischaemia-reperfusion injury (85-93). It seems to be likely that the cardioprotective effects of

MLA involves myocardial iNOS enzyme activation (90) which can be blocked by aminoguanidine (91). The involvement of iNOS with subsequent activation of myocardial K^+_{ATP} channels (86) is also purposed.

Delayed protection of the myocardium possibly requires synthesis of various proteins, and several transcriptional pathways are stimulated by preconditioning (94-97). The transcription factor which is involved in the expression of iNOS is nuclear factor κ-B (NFκB). The induction of the iNOS by certain stress stimuli depends on the activation of this transcription factor (98). We have examined whether NFκB is activated after rapid cardiac pacing in dogs, and in rabbits subjected to ischaemic preconditioning (IP). In dogs right ventricular pacing increased the activity of the p65 subunit 1 h later (Figure 21). In rabbits brief episodes of coronary artery occlusion resulted in a more rapid activation of NFκB; both p65 and p50 subunits were activated 10 min after ischaemic preconditioning (Figure 21). This might indicate species difference but also that coronary artery occlusion is stronger stimulus for evoking the protection than cardiac pacing. In our experiments in the rabbit heart the NFκB starts to be downregulated 1 h or 3 h after preconditioning. A similar time course of the activation of NFκB has been found in conscious rabbits (99). However, Bolli and his colleagues have shown the maximal activity of the factor 30 min after ischaemic preconditioning and the downregulation of the factor was seen 2 and 4 h later (99).

Other hypotheses for the mechanism of delayed cardioprotection suppose "de novo" synthesis of cytoprotective proteins such as heat stress proteins (100-102) or antioxidant enzymes (103-105). There is strong evidence that the stress proteins, particularly of the hsp70 and hsp27 families, play an important role in defence mechanisms against ischaemic injury (102). These proteins act as molecular chaperones and prevent the denaturation of proteins resulting from various stress stimulus in the cells (106). The results of studies which aimed to examine the role of antioxidant enzymes such as superoxide dismutase (Mn-SOD) or catalase in this protection are controversial. In pigs, the protection against contractile dysfunction which is observed 24 h after ischaemic preconditioning was not associated with an increase in the protein level, or activity, of antioxidant enzymes (107). However, in cultured rat cardiac myocytes hypoxic preconditioning or heat stress resulted in tolerance to sustained ischaemia 24 h later with a concomitant increase in the level and the activity of Mn-SOD (108, 109). Hoshida and colleagues have demonstrated that this delayed protection against ischaemic injury was due to enhanced Mn-SOD activity in the canine heart, 24 h after ischaemic preconditioning (110).

4.4. Clinical relevance of ischaemic preconditioning

There is no doubt that the powerful protection afforded by preconditioning against the severe consequences of a sustained ischaemic episode, such as sudden cardiac death resulting from fatal ventricular fibrillation or acute myocardial infarction, has great clinical relevance. The fact that short episode of ischaemia can be protective may offer possibilities for the treatment of ischaemic heart disease. For example, those patients who have an episode of angina (short ischaemia) resulting from ischaemic heart disease prior to myocardial infarction have a lower mortality rate and reduced necrosis (111, 112). This phenomenon suggests that the human heart can adapt rapidly to an ischaemic stress. One example of preconditioning in the human myocardium might be interventions during PTCA. Short episodes of ischaemia evoked by repeated balloon inflation resulted in significantly less lactate production and ST-segment changes and the patients reported less pain (17, 113).

It is hoped that a more intensive research related to the mechanism of this prolonged protection associated with preconditioning will advance our knowledge and understanding of this from of cardioadaptation and eventually point to new cardioprotective strategies.

5. New findings

1. We have demonstrated for first time that the marked delayed protection of the myocardium induced by cardiac pacing, which is very powerful 24 h later but fades 48 h after the pacing stimulus, can be extended for a longer time (to 48 h or 72 h). After repeated pacing there is a pronounced and extended duration of protection against ischaemia and reperfusion-induced ventricular arrhythmias; this prolonged protection starts to fade only 96 h after repeated pacing.

2. We have also shown that both nitric oxide and prostacyclin are important mediators of the antiarrhythmic protection induced by ischaemic preconditioning. This antiarrhythmic protection was completely abolished by simultaneous blockade of L-Arg/nitric oxide and cyclooxygenase pathways.

3. Delayed antiarrhythmic protection induced by single or repeated right ventricular pacing may involve an increased nitric oxide production, most probably by the induced form of nitric oxide synthase. This is supposed by those experiments in which the protection was markedly attenuated after administration of aminoguanidine and AEST, two selective inhibitors of iNOS.

4. We have found an increased activity of NFκB, a transcription factor involved in the expression of iNOS, 1 h after rapid cardiac pacing and 10 min after ischaemic preconditioning. This also supports the hypothesis of the role of iNOS in delayed cardioprotection.

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7. Acknowledgements

I would like to thank Professor Julius Gy. Papp for giving me the opportunity to spend 6 years and carry out my studies in the Department of Pharmacology and Pharmacotherapy, Albert Szent-Györgyi Medical University, as an undergraduate and postgraduate student.

I would like to express my deepest respect and gratefulness to my friend and supervisor Professor Ágnes Végh for her endless patience and for her guidance with excellent scientific knowledge in my studies.

I would like to thank Professor James R. Parratt, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow for his great help in my publications and for his kind support over the past years.

I also thank all of my colleagues Erika Tóth, Gábor Girst, Ágnes Krajevszki, Andrea Ábrahám and Mária Kosztka for their help to carry out my experiments.

My acknowledgement is due to Professor Derek M. Yellon and Gary F. Baxter for giving me the opportunity to spend a few months in the Hatter Institute for Cardiovascular Studies, University College London, and for their support in my work.

A special thank is due to my family for their help, patience and understanding, without whom I wouldn't have been able to finish my PhD studies.